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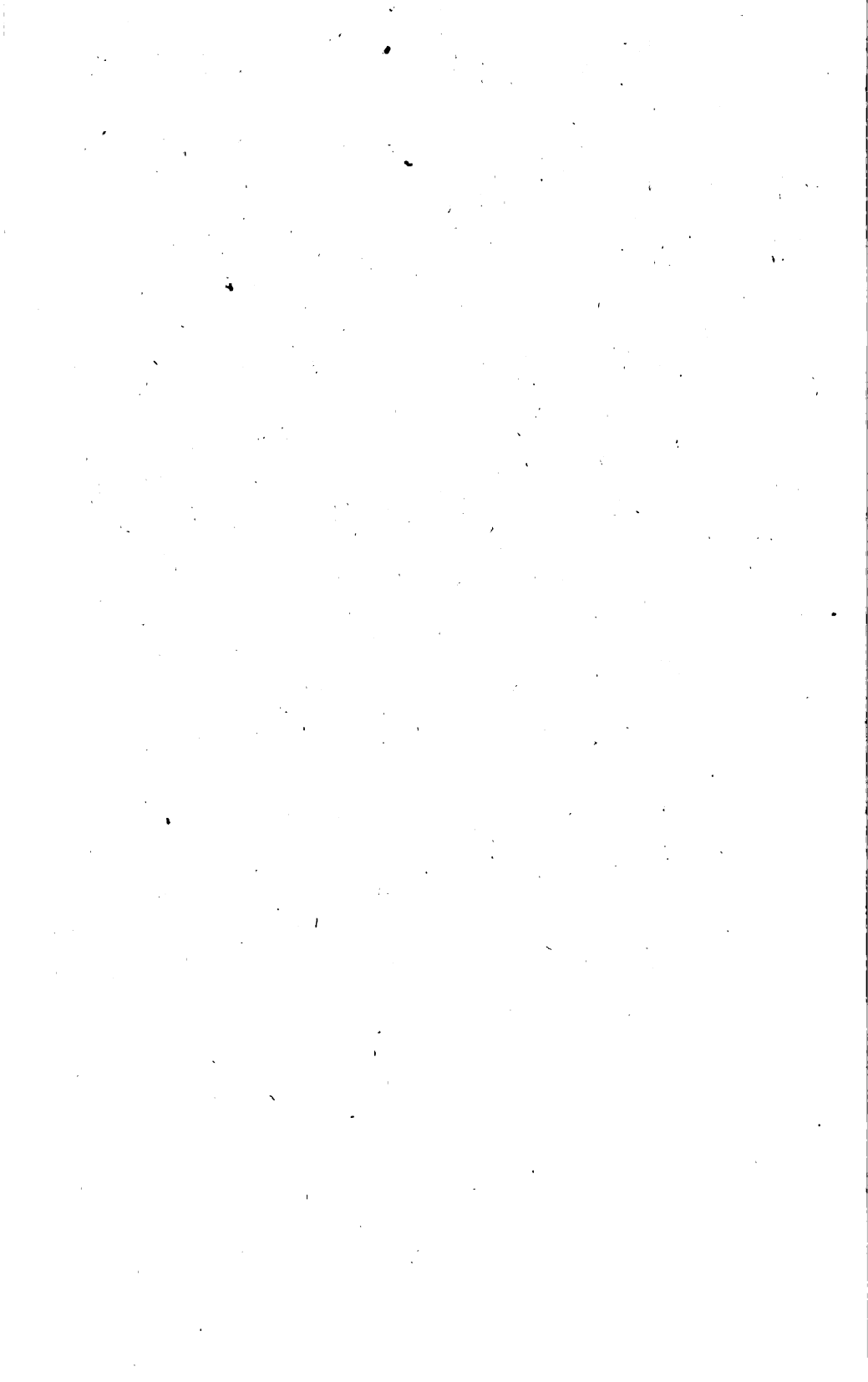


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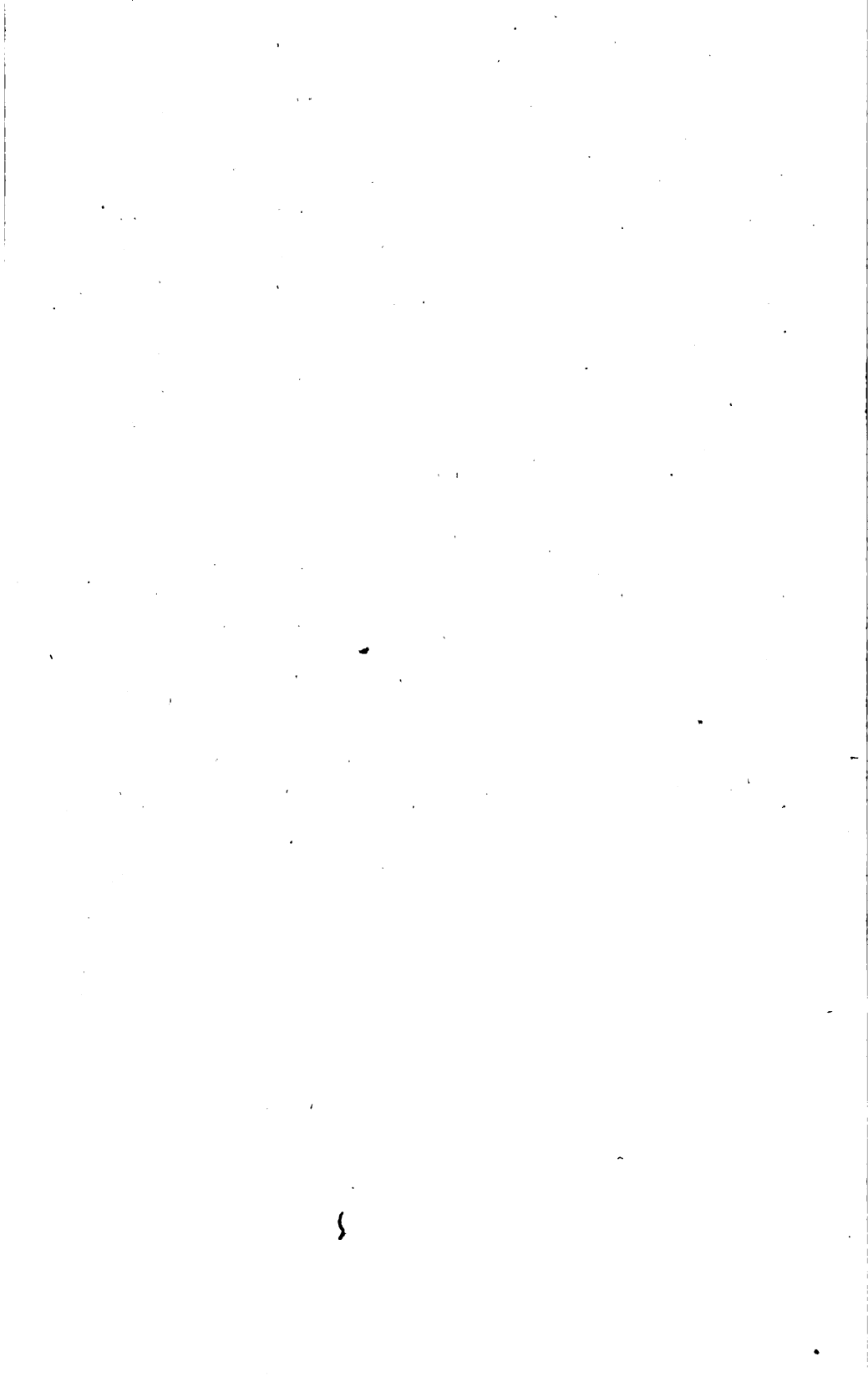


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THE COPPER COMPLEXES OF AMINO-ACIDS, PEPTIDES AND PEPTONES.¹

FIRST PAPER.

By P. A. KOBER AND K. SUGIURA.

(From the Harriman Research Laboratory, Roosevelt Hospital, New York City.)

(Received for publication, August 8, 1912.)

INTRODUCTION.

That proteins and their constituents combine with heavy metals to form "complexes" has been known for some time. Among these copper has received the most attention and considerable work has been done on the copper complexes of amino-acids; they have been isolated,² analyzed and studied by chemical and physical methods. On the other hand the complexes of peptides and peptones have received only a superficial and qualitative study. This is not due to the unimportance of peptide copper salts, for the behavior of peptide and peptone copper salts on treatment with an excess of alkali has been and is yet the most reliable test known for protein-like substances. The real reason for this lack of investigation is, without doubt, due first to the difficulty of obtaining peptides and peptones pure, and second to the fact that the copper salts of these substances are not easily crystallized. It is our belief that a quantitative study of these copper salts³ will throw some light on the constitution of proteins and it is therefore our intention to continue from time to time our efforts in this field.

In a previous preliminary communication⁴ one of us showed a

¹ Read before the Section on Organic Chemistry, International Congress of Applied Chemistry, New York, Sept., 1912.

² Ley: *Zeitschr. f. Electrochem.*, xi, p. 954; Schiff: *Ann. d. Chem.*, xliii, p. 123; Bruin: *Chem. Zentralblatt*, p. 824, 1904; Ley and Krafft: *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 697, 1907; Fischer: *Untersuchungen über Amino-säuren, Polypeptide und Proteine*, 1906.

³ The term *copper salt*, used here and throughout the paper, has reference to a true salt, i.e., the product of the action of amino-acids and protein-like substances in general on cupric hydroxide, oxide or carbonate.

⁴ Kober: *this Journal*; x, p. 9, 1911.

striking difference between the salts of amino-acids on the one hand, and the salts of peptides and peptones on the other. As stated in the previous paper, "the copper salts of the amino-acids in alkaline solutions, (. contrary to statements in scientific literature,) particularly on warming or on boiling, precipitate copper as the hydroxide, quantitatively. Peptides and peptones, on the other hand, give very little or no hydroxide under the same conditions."

The explanation of this important difference may possibly be due to one or more of the following reasons: (1) A marked difference in the constitution of these salts; (2) A change of constitution brought about by the excess alkali; (3) A change in the form or condition of the copper due to the presence of the protein-like substances, *e.g.*, "colloidal copper."⁶

The fact that peptides do not form copper salts strictly in accordance with the number of free carboxyl groups,⁶ as do most of the amino-acids, favors the theory of a difference in the constitution as the cause of their abnormal behavior on treatment with excess alkali. The fact that most copper salts of peptides and peptones change color on treatment with alkali, giving the so-called "biuret color," indicates that a change of structure in these peptides takes place on adding excess alkali. The fact that a similar phenomenon described by Paal and Leuze⁷ was explained as a "colloidal process" gives a certain degree of plausibility to the third reason. As will be seen, the data given in the latter part of this paper support the first two explanations, while not many facts support the third.⁸

As Leuchs,⁹ conjunctively with Manasse and La Forge, has considerable experimental basis for considering that carbethoxyl-glycyl-glycine ester and allied compounds have two forms, the lactim and the lactam, we believed at first that the lactim form was the chief cause for the copper not precipitating in alkaline solutions, behaving in this respect like other hydroxy-acids, such

⁶ Paal and Leuze: *Ber. d. deutsch. chem. Gesellschaft.*, xxxix, p. 1545.

⁶ Fischer: *Untersuchungen über Amino-säuren, Polypeptide und Proteine*, 1906, p. 50.

⁷ *Loc. cit.*

⁸ Copper salts in weak alkaline solutions dialyze, but when the alkali is strong, they combine with the membrane and cause difficulties.

⁹ *Ber. d. deutsch. chem. Gesellschaft.*, xl, p. 3235; xli, p. 2586.

as lactic, tartaric, etc.; but owing to the lack of spectrographic data we cannot say whether this really plays a part or not.

As part of our results are at variance with some obtained previously by other investigators, our technique and results will be described and discussed before data are weighed in support of any theory.

TECHNIQUE.

Fischer,¹⁰ Abderhalden,¹¹ and others prepared the copper salts of amino-acids, peptides and peptones by boiling with cupric oxide. We found that they were formed best at a low temperature, with cupric hydroxide, preferably in an ice mixture. Since some heat of neutralization makes the process an exothermal one, it is not unexpected that the reaction will be complete at a low temperature. A further objection to boiling lies in the fact that a few of these salts have a slight tendency to hydrolyze. Where a reaction can be brought about at a freezing temperature the danger of decomposing unstable peptides is obviously reduced to a minimum.

The form of the copper hydroxide is very important as on standing it is dehydrated with the formation of cupric oxide. That cupric oxide is slower in its reaction with these peptides can readily be seen from the constitution of the copper salts.¹²

The best conditions for forming these copper salts quantitatively are as follows:

a. For soluble substances.

For every 0.1 gram of amino-acid or peptide 5 cc. of 5 per cent cupric chloride solution are diluted to 150 or 200 cc. with ice-cold distilled water and neutralized with alkali. This can be done most suitably by mixing 100 grams of fine ice with 5 cc. of 5 per cent cupric chloride solution and 100 cc. of distilled water and neutralizing with $\frac{N}{5}$ alkali, using phenolphthalein as an indicator. The neutralized mixture is then filtered, the ice being placed on the filter also in order to keep the precipitated cupric hydroxide cool. The hydroxide is washed once or twice with cold distilled water on the filter paper, removed with the ice to the cooled 10-20 cc. solution of the amino-acid or peptide and stirred for from five to ten minutes.

On filtering off the excess cupric hydroxide and washing thoroughly, the solution will contain the copper salts of all the substances. As a rule, the solutions require boiling, to decompose the very appreciable amount

¹⁰ Fischer: *loc. cit.*

¹¹ Abderhalden: *Ber. d. deutsch. chem. Gesellsch.*, xxxx, p. 2746.

¹² *Amer. Chem. Journ.*, Nov., 1912.

of carbamino salts that are formed with CO_2 of the air, and this may be done before filtering. To ascertain the amount of copper hydroxide dissolved, the copper salt may be titrated directly (with 0.04 N $\text{Na}_2\text{S}_2\text{O}_3$) by adding 5-10 cc. of glacial acetic acid and 2-4 grams KI. The amount of carbamino salts formed is increased by the presence of sodium chloride, whereas alcohol, on the contrary, hinders their formation.

b. For insoluble substances with slightly soluble copper salts.

If the amino-acid or peptide is insoluble, 10-20 cc. of $\frac{N}{10}$ ammonia are added to the sample and it is stirred until dissolved. If necessary the mixture may be heated to hasten solution. After cooling the solution in an ice mixture, cold cupric hydroxide, made as above, is added and stirred for from five to ten minutes; 150 cc. of water are then added and the solution filtered after thorough shaking. The precipitate is washed well with hot water and the filtrate and wash waters are concentrated. The amount of copper hydroxide dissolved may be titrated iodimetrically in the usual way. Controls on this method, using copper hydroxide and 20 cc. of $\frac{N}{10}$ ammonia, gave only 0.0003 to 0.0005 gram of copper oxide in the filtrate.

c. For insoluble substances with quite insoluble copper salts.

Where the copper salt crystallizes out and is filtered off with the excess copper hydroxide, it is necessary to separate the two precipitates.

Very satisfactory reagents for this purpose are the bicarbonates of sodium and potassium. Using 10 and 20 per cent solutions of KHCO_3 we have obtained the results given below on leucine, tryptophane, cystine, amino-*n*-caproic acid and phenylglycine.

The amino substance is treated as in *b*, but the excess copper hydroxide, mixed with the insoluble copper salt, is then treated with 20 cc. of 20 per cent KHCO_3 and washed with small lots of 10 per cent KHCO_3 until the filtrate shows only traces of copper. To determine directly the amount of copper hydroxide dissolved, the final residue of copper salt is then transferred with the filter paper to the first filtrate and after being dissolved in dilute hydrochloric¹³ acid is titrated as before.

On page 13 under experimental notes we give a few experiments on the solubility of these "insoluble" copper salts in various concentrations of potassium bicarbonate. In the near future we hope to give the details of more experiments along these lines, especially on the determination of amino-acids in the presence of polypeptides.

Boiling directly with copper hydroxide or oxide will result without doubt in the incomplete formation of copper salts; this fact helps to explain the unexpected results obtained by Abderhalden and Hirsch on *d*-alanyl-*l*-leucyl-isoleucine and its glycyl derivatives (see page 7).

¹³ As large amounts of potassium acetate retard the iodimetric titrations of copper, we neutralize with pure HCl.

SUMMARY OF DATA.

Amino-acid copper salts.

The results with our technique on α -amino-acids confirm the analyses made by previous investigators of isolated copper salts, which have without exception the general formula CuA_2 , where A represents one molecule of monobasic α -amino-acid.

A few examples will suffice:

Monobasic amino-acids.

SUBSTANCES	METHOD OF PREPARATION	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N. NAOH (CO_2 FREE) AND BOILING	CuO IN PRECIPITATE AFTER ADDING 3 CC. N. NAOH (CO_2 FREE) AND BOILING	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENTAGE OF THEORETICAL
		grams	grams	grams	grams	per cent
Glycine.....	a	0.1015	0.0000	0.0536	0.0538	99.6
Alanine.....	a	0.1019	0.0000	0.0453	0.0455	99.6
Aminobutyric acid.....	b	0.1022	0.0004	0.0403	0.0395	102.0
Active valine.....	a	0.0944	0.0015	0.0301	0.0321	93.8
Leucine.....	c	0.1010	0.0054	0.0361	0.0307	102.6
Normal aminocaproic acid.....	c	0.1003	0.0002	0.0315	0.0305	105.7
Isoleucine.....	a	0.1014	0.0011	0.0307	0.0308	103.2
Active proline.....	a	0.1018	0.0078	0.0269	0.0352	98.6
Phenylalanine.....	b	0.1001	0.0000	0.0244	0.0242	100.8
Phenylglycine.....	c	0.1011	0.0000	0.0280	0.0266	105.3
Tyrosine.....	b	0.1027	0.0007	0.0263	0.0226	116.4
Tryptophane*.....	b	0.1022	0.0000	0.0183	0.0199	92.0
Asparagine.....	b	0.1005	0.0005	0.0308	0.0303	101.7
Sarcosine hydrochloride†.....	a	0.1002	0.0021	0.0299	0.0315	101.6
Lysine picrate‡.....	a	0.1006	0.0006	0.0097	0.0107	96.3
Arginine dinitrate.....	a	0.1010	0.0011	0.0121	0.0134	98.5
Histidine dihydrochloride§.....	a	0.1000	0.0161	0.0005	0.0175	94.9

*If more than 0.1 gram tryptophane is used, method c must be added to this technique.

†All acid salts, such as hydrochlorides, nitrates, etc., are neutralized with $\frac{N}{10}$ alkali, using phenolphthalein as an indicator, before treating with copper hydroxide as in method a.

‡Our thanks are due to Dr. Levene of the Rockefeller Institute for furnishing us with isoleucine, arginine and lysine.

§Histidine forms a complex salt, as do the other monobasic amino-acids, and on treatment with excess alkali, changes its color but little. Only on boiling the color changes towards a biuret. It is not a clear color, but smoky, and makes the solution look very dark. Characteristic is the deep red color to which the alkaline solution turns on the addition of acid. We expect to make this a basis for the colorimetric qualitative and quantitative estimation of histidine.

Peptide Copper Complexes

With other amino-acids, whose NH_2 group is in the β -position, Fischer found the formula to be CuA_2 , where A is monobasic amino-acid, except (as in the case of isoserine) when an oxy group is in the α -position, where the formula is CuA .

We have obtained the same results with isoserine,¹⁴ using the technique a:

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N NaOH (CO_2 FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N NaOH (CO_2 FREE) AND BOILING	TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
	grams	grams	grams	grams	grams	per cent
Iso-serine.....	0.1010			0.0807	0.0765	105.5
Iso-serine.....	0.1009	0.0299	0.0499	0.0798	0.0764	104.5

According to Fischer,¹⁵ when the amino group is in the γ -, δ -, or ϵ -position (regardless of an hydroxy group in the α position) no copper salts are formed.

Our results confirm the previous figures on dibasic salts having a general formula CuA , where A is a dibasic acid, such as aspartic, glutaminic, cystinic, etc.

Dibasic amino-acids.

SUBSTANCE	METHOD OF PREPARATION	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N NaOH (CO_2 FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N NaOH (CO_2 FREE) AND BOILING	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
		grams	grams	grams	grams	per cent
Cystine.....	c	0.1008	0.0000	0.0331	0.0334	99.1
Aspartic acid*.....	a	0.0997	0.0009	0.0579	0.0596	98.6
Glutaminic acid*.....	a	0.1025	0.0004	0.0518	0.0554	94.9

*Before boiling the solutions of copper salts are diluted to 150 cc. with water.

¹⁴ Made according to Fischer's directions from epichlorhydrin. *Ber. d. deutsch. chem. Gesellsch.*, xxxv, p. 3787.

¹⁵ E. Fischer and L. Zemplen: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 4883, 1909.

Polypeptide copper salts.

Out of a hundred or more polypeptides, Fischer, Abderhalden and their collaborators have analyzed only about eight of the polypeptide copper salts; and these, with three exceptions (nos. VI, VII and VIII) were insoluble and crystallized out. In such cases there can be no doubt as to the purity of the product and the results are confirmed by our work on the more soluble copper peptides. These cases are as follows:

I. (Leucyl-glycine-copper)₂O + H₂O¹⁶ or (C₈H₁₅O₃N₂Cu)₂O + H₂O.

Fischer concluded that one molecule of copper hydroxide combined with one molecule of leucyl-glycine and that two molecules of the copper-leucyl-glycine are connected by an oxygen atom. Although we have found one molecule of copper hydroxide to one of leucyl-glycine, we do not believe that an oxygen atom connects two molecules of copper-leucyl-glycine. We have determined the molecular weight of this salt by the cryoscopic method, and find it to have a molecular weight consistent with the formula leucyl-glycine-Cu;

II. (Phenyl-glycine-glycine)₁copper,¹⁷ C₁₀H₁₀N₂O₃Cu.

III. (*l*-Leucyl-*l*-histidine)₁copper,¹⁸ C₁₂H₁₃O₃N₄Cu.

IV. (Alanyl-*l*-tryptophane)₁copper,¹⁹ C₁₄H₁₅N₃O₃Cu.

V. (*l*-Prolyl-*l*-phenylalanine)₁copper,²⁰



VI. (*d*-Alanyl-*l*-leucyl-isoleucine)₂copper,²¹ (C₁₅H₂₇O₄N₃)₂Cu.

VII. (Glycyl-*d*-alanyl-*l*-leucyl-isoleucine)₂copper,²²

(peptide)₂Cu.

VIII. (Tri-glycyl-glycine)₂copper,²³ (tetrapeptide)₂Cu.

Out of the large number of peptide copper salts examined by Fischer, Abderhalden and ourselves, the last three form an apparent exception to the rule. There are good reasons to believe that

¹⁶ Fischer: *Ann. d. Chem.*, cccxl, p. 145.

¹⁷ Fischer: *ibid.*, p. 195.

¹⁸ Fischer and L. H. Cone: *ibid.*, cccxlxiii, p. 107.

¹⁹ Abderhalden and M. Kempe: *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 2737.

²⁰ Fischer: *ibid.*, xlii, p. 4752.

²¹ Abderhalden and Hirsch: *ibid.*, xliii, p. 2439.

²² *Ibid.*

²³ Curtius: *ibid.*, xxxvii, p. 1294.

the formulas given for VI, VII and VIII do not represent true copper salts, as the methods used in each case are no guarantee of the complete formation of the salts.

Using the technique described above, we have formed the copper salts of the following peptides, and have found the results to be consistent with the formula, (peptide)₁Cu₁.

Dipeptides.

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N. NaOH (CO ₂ FREE) AND BOIL- ING	CuO IN PRECIPITATE AF- TER ADDING 3 CC. N. NaOH (CO ₂ FREE) AND BOILING	TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
	grams	grams	grams	grams	grams	per cent
Glycyl-glycine.....	0.1123	0.0536	0.0070	0.0606	0.0616	98.4
Glycyl-alanine.....	0.1006	0.0501	0.0049	0.0550	0.0548	100.4
Glycyl-d-alanine.....	0.1011	0.0524	0.0006	0.0530	0.0550	96.5
Glycyl-aminobutyric acid.....	0.1007	0.0437	0.0030	0.0467	0.0500	93.4
Glycyl-valine.....	0.1012	0.0400	0.0062	0.0462	0.0462	100.0
Glycyl-d-valine.....	0.1015	0.0390	0.0059	0.0449	0.0464	96.8
Glycyl-leucine.....	0.1005	0.0380	0.0022	0.0402	0.0425	94.6
Glycyl-l-leucine.....	0.1006	0.0370	0.0036	0.0406	0.0425	95.5
Glycyl-amino-n-cap- roic acid.....	0.0963	0.0377	0.0014	0.0391	0.0407	96.1
Glycyl-asparagine.....	0.1028	0.0397	0.0031	0.0428	0.0433	98.8
Glycyl-phenylglycine.....	0.1021	0.0355	0.0030	0.0385	0.0390	98.7
Glycyl-d-phenylglycine.....	0.1022	0.0358	0.0010	0.0368	0.0391	94.1
Glycyl-tyrosine.....	0.0545	0.0142	0.0043	0.0185	0.0181	102.2
Glycyl-tryptophane.....	0.1016	0.0257	0.0010	0.0267	0.0310	86.1
Alanyl-glycine.....	0.1007	0.0516	0.0032	0.0548	0.0548	100.0
l-Alanyl-glycine.....	0.1005	0.0496	0.0054	0.0550	0.0547	100.5
d-Alanyl-d-alanine.....	0.1001	0.0465	0.0041	0.0506	0.0497	101.8
Aminobuteryl-glycine.....	0.1010	0.0467	0.0045	0.0512	0.0502	102.0
Valyl-glycine.....	0.1005	0.0410	0.0059	0.0469	0.0459	102.2
Leucyl-glycine.....	0.0901	0.0344	0.0041	0.0385	0.0381	101.0
Leucyl-leucine.....	0.1006	0.0288	0.0014	0.0302	0.0328	92.1
l-Leucyl-d-leucine.....	0.1004	0.0266	0.0021	0.0287	0.0327	87.8
d-Leucyl-l-leucine.....	0.1011	0.0268	0.0003	0.0271	0.0330	82.1
d-Leucyl-d-leucine.....	0.1007	0.0294	0.0013	0.0307	0.0328	93.6
Amino-n-caproic-gly- cine.....	0.0664	0.0257	0.0015	0.0272	0.0281	96.8
Leucyl-asparagine.....	0.1004	0.0205	0.0010	0.0215	0.0326	66.0

*Tripeptides.*²⁴

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC. N NaOH (CO ₂ FREE) AND BOIL- ING			CuO IN PRECIPITATE AF- TER ADDING 3 CC. N NaOH (CO ₂ FREE) AND BOILING			TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	CuO IN FILTRATE AS PERCENTAGE OF THEO- RETICAL	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
	grams	grams	grams	grams	grams	grams	grams	grams	per cent	per cent	
Glycyl-glycyl-glycine.....	0.1003	0.0370	0.0006	0.0376	0.0422	87.7	89.1				
Glycyl-glycyl-alanine.....	0.1021	0.0356	0.0064	0.0420	0.0400	89.0	105.0				
Glycyl-glycyl-aminobutyric acid.....	0.1011	0.0382	0.0006	0.0388	0.0371	103.0	104.6				
Glycyl-glycyl-valine.....	0.0516	0.0155	0.0005	0.0160	0.0178	87.1	89.9				
Glycyl-glycyl-leucine.....	0.1010	0.0279	0.0044	0.0323	0.0328	86.4	98.5				
Glycyl-glycyl-l-leucine.....	0.1020	0.0287	0.0024	0.0311	0.0331	86.7	94.0				
Glycyl-d-alanyl-d-alanine...	0.0536	0.0144	0.0001	0.0145	0.0192	75.0	75.5				
Glycyl-leucyl-glycine.....	0.1013	0.0293	0.0004	0.0297	0.0329	88.5	90.3				
Alanyl-glycyl-glycine.....	0.0987	0.0254	0.0031	0.0285	0.0387	65.6	73.6				
d-Alanyl-glycyl-glycine.....	0.0492	0.0168	0.0014	0.0182	0.0193	87.0	94.3				
Alanyl-leucyl-glycine.....	0.1012	0.0274	0.0037	0.0311	0.0311	88.1	100.0				
Amino-buteryl-glycyl-glycine	0.1011	0.0350	0.0028	0.0378	0.0371	94.3	101.9				
Valyl-glycyl-glycine.....	0.1023	0.0288	0.0027	0.0315	0.0352	81.8	89.5				
Leucyl-glycyl-glycine.....	0.1013	0.0283	0.0057	0.0340	0.0329	86.1	103.3				
l-Leucyl-glycyl-glycine.....	0.1011	0.0284	0.0043	0.0327	0.0328	86.6	99.7				
l-Leucyl-glycyl-d-alanine...	0.0569	0.0161	0.0008	0.0169	0.0175	92.0	96.6				
Leucyl-alanyl-glycine.....	0.1002	0.0289	0.0040	0.0329	0.0308	93.8	106.8				
Leucyl-alanyl-alanine.....	0.1017	0.0272	0.0050	0.0322	0.0296	91.9	108.8				
l-Leucyl-d-alanyl-d-alanine..	0.1014	0.0262	0.0033	0.0295	0.0295	88.8	100.0				
Amino-n-caproyl-glycyl- glycine.....	0.1003	0.0304	0.0035	0.0339	0.0325	93.5	104.3				

²⁴ The majority of the peptides used in this study were prepared or collected by the late Dr. Arthur H. Koelker. A small number (four dipeptides and three tripeptides) were made according to Fischer's directions, by Dr. H. Hager and ourselves, in this laboratory. Glycyl-tryptophane was obtained in very pure crystalline form through the kindness of Kalle and Co.

Peptide Copper Complexes

Tetrapeptides.

SUBSTANCE	WEIGHT OF SAMPLE		CuO IN FILTRATE AFTER ADDING 3 CC. N. NAOH (CO ₂ FREE) AND BOIL- ING		CuO IN PRECIPITATE AP- PEARING 3 CC. N. NAOH (CO ₂ FREE) AND BOILING		TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	CuO IN FILTRATE AS PERCENTAGE OF THEO- RETICAL	TOTAL CuO AS PERCENT- AGE OF THEORETICAL
	grams	grams	grams	grams	grams	grams	grams	per cent	per cent	
Alanyl-diglycyl-glycine.....	0.1008	0.0267	0.0017	0.0284	0.0308			86.7	92.2	
Aminobuteryl-diglycyl-glyc- ine.....	0.1050	0.0260	0.0037	0.0297	0.0305			85.2	94.1	
Leucyl-diglycyl-glycine*.....	0.1008	0.0239	0.0030	0.0269	0.0265			90.2	101.5	
n-Amino-caproyl-diglycyl- glycine.....	0.1017	0.0202	0.0005	0.0207	0.0268			75.4	77.2	

* Filtered without boiling to decompose carbamino salts.

Using our same technique it was of interest to see the amount of copper hydroxide dissolved by peptones.

Peptones, etc.

SUBSTANCE	WEIGHT OF SAMPLE		CuO IN FILTRATE AFTER ADDING 3 CC. N. NAOH (CO ₂ FREE) AND BOIL- ING		CuO IN PRECIPITATE AP- PEARING 3 CC. N. NAOH (CO ₂ FREE) AND BOILING		MOLECULAR WEIGHT* CAL- CULATED FROM TOTAL CuO
	grams	grams	grams	grams	grams	grams	
"Roche" peptone.....	0.1110	0.0302	0.0173				186
"Ereptone".....	0.1005	0.0064	0.0133				406
"Merck" peptone.....	0.1020	0.0044	0.0002				1765
"Witte's" peptone.....	0.1025	0.0041	0.0000				1990
"Witte's" peptone.....	0.1046	0.0042	0.0000				1982

* This calculation is based on the assumption that one molecule of peptone, as in the case of the peptides, combined with only one molecule of copper hydroxide.

The results on the peptides show unmistakably that one molecule of peptide, whatever number of amino-acids it may contain, combines with only one molecule of copper hydroxide. This interest-

ing fact will no doubt give us an easy method of determining the molecular weights of peptides, provided the copper salts can be separated from the excess copper hydroxide.

The questions that remain to be solved, namely, how are these copper salts formed and what is their structure, will be taken up in a separate paper,²⁵ in conjunction with the biuret configuration.

SUMMARY.

1. We have developed a technique for making, quantitatively, copper salts of (a) soluble amino-acids and peptides; (b) insoluble amino-acids having soluble copper salts; (c) insoluble amino-acids having insoluble copper salts.

2. We have formed copper salts of twenty-six dipeptides, twenty tripeptides and four tetrapeptides, quantitatively, in solution, and have found the results to be consistent with the formula, (peptide)₁Cu. This supports the work of Fischer and Abderhalden on five isolated salts.

3. We have found: (a) That on an average 99 per cent of the copper of all amino-acid salts (except that of histidine) is precipitated as oxide when treated with a certain excess of alkali.²⁶

(b) That on an average 6.4 per cent of the copper of dipeptide salts is precipitated as oxide with the same excess of alkali.

(c) That on an average 6.3 per cent of the copper of tripeptide²⁷ salts is precipitated as oxide with the same excess of alkali.

(d) That on an average 7.3 per cent of the copper of tetrapeptide salts is precipitated as oxide with the same excess of alkali.

Our thanks are due to Dr. Wm. G. Lyle for his encouragement in this work, and for placing at our disposal the excellent collection of peptides and amino-acids belonging to the late Dr. A. H. Koelker, and to Miss Calm M. Hoke for much assistance in preparing this article for publication.

²⁵ *Amer. Chem. Journ.*, Nov., 1912.

²⁶ Using 3-5 cc. N CO₂-free NaOH for every 0.1 gram substance and boiling.

²⁷ Assuming all the substances to be perfectly pure, which of course they are not. It is probable that perfectly pure tri- and tetra-peptides will not precipitate any of the copper of their salts under the conditions given above. We are convinced that impurities will account for most of the precipitation.

Peptide Copper Complexes

EXPERIMENTAL NOTES.

a. Copper oxide as a reagent.

In our first work on copper salts we used black copper oxide obtained in the open market as a reagent, but found that with peptides it reacted too slowly. Then we tried the commercial basic carbonate, and found it more useful, but not quite satisfactory. *Freshly prepared copper hydroxide, however, was found not only efficient but almost instantaneous in its reaction at 0°C.* The amount of surface is, of course, a factor in the reaction, and therefore a gelatinous precipitate is, other things being equal, preferable to a coarse granular precipitate.

As Fischer and Abderhalden used suspended copper oxide in making their salts, it was necessary to investigate the relative efficiency of this reagent on various amino-acids and peptides *when boiled*.

The following table gives the results with *freshly made copper hydroxide and freshly made copper oxide suspensions on boiling*. The latter were made by allowing a suspension of the hydroxide to stand several days in the laboratory, during which time it changed its color from blue to almost black.

SUBSTANCE	WEIGHT OF SAMPLE	CUO DISSOLVED AFTER BOILING 10 MINUTES WITH $\text{Cu}(\text{OH})_2$	CUO DISSOLVED AFTER BOILING 10 MINUTES WITH CuO	THEORETICAL WEIGHT OF CuO	CUO AS FOUND TO THEORETICAL
	grams	grams	grams	grams	per cent
Glycine.....	0.1011	0.0564		0.0536	105.2
Glycine.....	0.1016		0.0558	0.0539	103.5
Alanine.....	0.1034	0.0482		0.0462	104.3
Alanine.....	0.1021		0.0472	0.0456	103.5
Alanyl-glycine.....	0.1013	0.0562		0.0551	103.0
Alanyl-glycine.....	0.1001		0.0515	0.0547	94.5
Leucyl-glycine.....	0.1023	0.0435		0.0433	100.5
Leucyl-glycine.....	0.1015		0.0435	0.0429	101.4
Alanyl-leucyl-glycine.....	0.1004	0.0210		0.0308	68.2
Alanyl-leucyl-glycine.....	0.1010		0.0137	0.0310	44.2
Glycyl-leucyl-glycine.....	0.1011	0.0242		0.0328	73.8
Glycyl-leucyl-glycine.....	0.1003		0.0150	0.0325	46.2
Alanyl-di-glycyl-glycine.....	0.1003		0.0195	0.0307	63.2

These results show that boiling, while suitable for amino-acids and dipeptides, cannot be used with tri- and tetra-peptides, and will easily account for the abnormal results obtained by Abderhalden and Hirsch.²⁸

b. Bicarbonates as solvents for copper hydroxide.

When cystine, tryptophane, leucine and other amino-acids, whose copper salts are insoluble, are treated as in method c (see p. 4), the copper salts are found almost wholly²⁹ in the precipitate, mixed with the excess copper hydroxide.

In order to show the effect of different concentrations of the bicarbonate on the solubility of copper hydroxide and the "insoluble" copper salts, the following preliminary experiments were made.

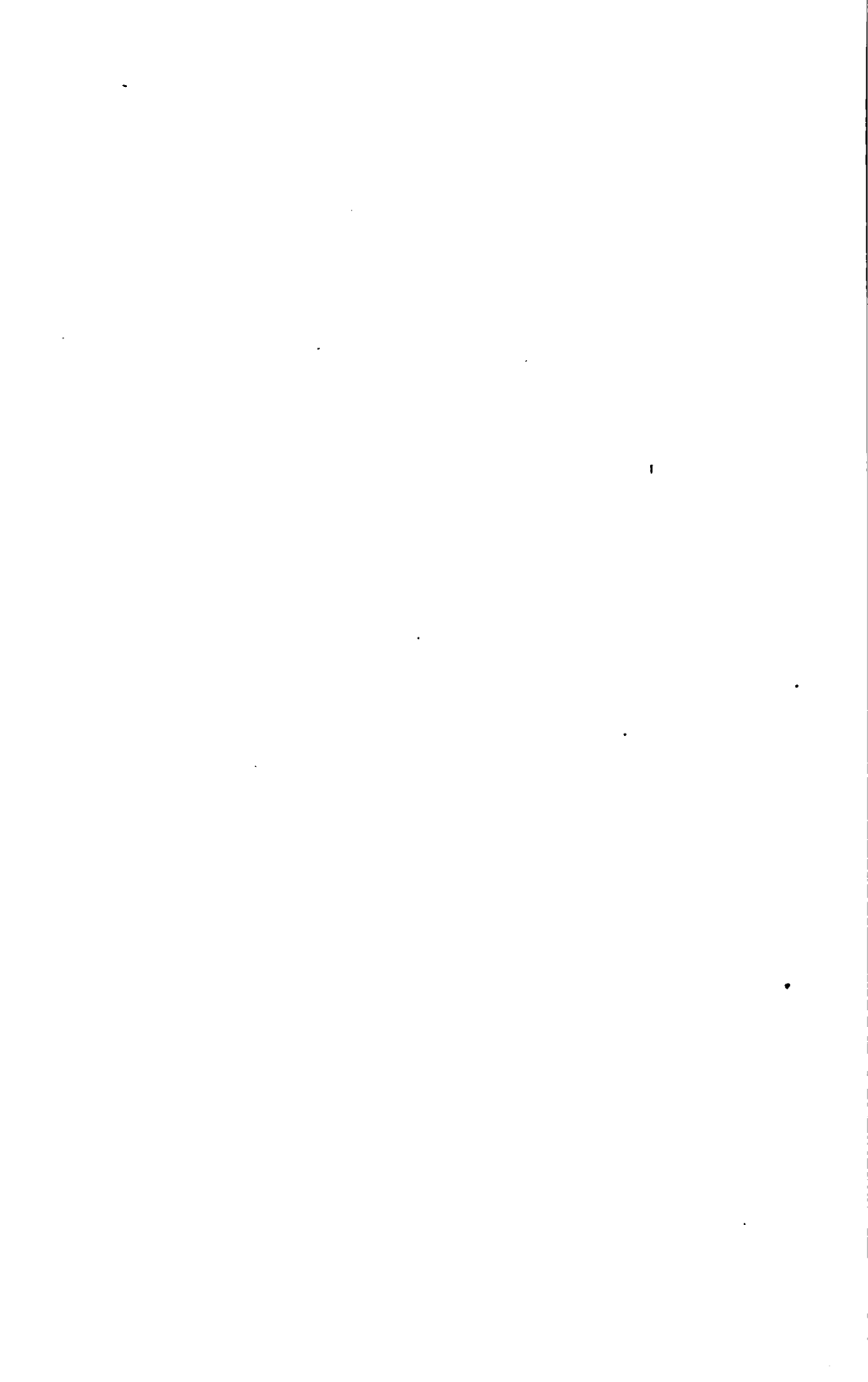
Pure copper salts of the amino-acids were made as described under c and a small portion (sufficient to leave a slight excess undissolved) was stirred constantly for five minutes in 25 cc. of 5 per cent, 10 per cent and 20 per cent potassium bicarbonate; controls were made on 25 cc. of distilled water.³⁰

SUBSTANCE	CuO DISSOLVED IN 25 cc. H ₂ O	CuO DISSOLVED IN 25 cc. 5 PER CENT KHCO ₃	CuO DISSOLVED IN 25 cc. 10 PER CENT KHCO ₃	CuO DISSOLVED IN 25 cc. 20 PER CENT KHCO ₃	CuO DISSOLVED IN 25 cc. 20 PER CENT KHCO ₃
	grams	grams	grams	grams	grams
Copper hydroxide.....	0.00000	0.0283	0.0580	0.0565	0.1680
Leucine, copper.....	0.00020	0.0006	0.0008		0.0015
n-Aminocaproic acid, cop- per.....	0.00003	0.0007	0.0016	0.0014	0.0060
Phenylglycine, copper....	0.00003	0.0006	0.0012	0.0013	0.0056
Tryptophane, copper.....	0.00020	0.0004	0.0004	0.0005	0.0007
Cystine, copper.....	0.00000	0.0000	0.0000	0.0000	0.0001

²⁸ *Loc. cit.*

²⁹ When less than 0.1 gram of tryptophane is used, the copper salt remains in a supersaturated condition in the filtrate and later crystallizes out.

³⁰ Only one concentration of sodium bicarbonate was used; the potassium bicarbonate, on account of its greater solubility, is preferable.



A STUDY OF THE MECHANISM OF PHLORHIZIN DIABETES.

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The distinctive feature of phlorhizin diabetes in contrast with other types is the significant diminution in the blood sugar content. The mechanism by which this condition of hypoglycaemia is established has been explained by a variety of theories, none of which, however, has received universal acceptance. Of the numerous views¹ that have been promulgated two have received considerable attention. It was the opinion of v. Mering² that the drug creates an increased permeability of the kidney for sugar, thus leading to the passage of blood sugar into the urine. To meet this drain upon the sugar of the blood an augmented activity of the blood sugar regulating mechanism is supposed to be set up whereby new sugar is formed in the body from some antecedent substance, presumably protein. If this explanation is correct one might fairly assume that extirpation or ligation of the kidneys in phlorhizin diabetes by preventing a renal loss of sugar would result in a restoration of sugar to its normal percentage in the blood. Minkowski's³ experiments tend to support this position although, as Levene⁴ pointed out, Minkowski obtained an increase of sugar in the blood beyond the normal after extirpation of the kidneys, which theoretically should not result if phlorhizin merely has a specific influence upon the kidney and does not act upon some other mechanism.

¹ For a discussion of the problem, cf. Macleod: *Recent Advances in Physiology and Bio-Chemistry*, Edited by Leonard Hill, 1906.

² v. Mering: *Verhandl. d. 5te Congresses f. inn. Med.*, 1886, p. 185.

³ Minkowski: *Arch. f. exp. Path. u. Pharm.*, xxxi, p. 85, 1893.

⁴ Levene: *Journ. of Physiol.*, xvii, p. 259, 1894-5.

A second view of the nature of phlorhizin diabetes, which was advocated by Pavy and others,⁵ differs from the first primarily in ascribing to the kidney the power of *producing* sugar. Under the influence of phlorhizin the cells of the renal tubules are supposed to exert a catabolizing action upon something reaching them from the blood, resulting in the liberation of dextrose in a manner comparable to that by which lactose is set free by the cells of the mammary gland.

That the conditions existing in pancreatic and phlorhizin diabetes are similar in many respects is well recognized. Thus in the starving dog the D:N ratios obtained in the two experimental states are somewhat alike. Moreover, it is generally conceded that in phlorhizin diabetes as well as in the condition induced by removal of the pancreas the ability of the organism to utilize dextrose may be somewhat diminished. The distinguishing difference between these two abnormal states is found in the lowered blood sugar content in phlorhizin diabetes and the existence of hyperglycaemia in pancreatic diabetes.

Although numerous investigations have been carried through concerning the relation of ligation or ablation of the kidneys to phlorhizin diabetes a review of the literature fails to reveal an experiment of this nature with animals in *total* diabetes, that is, with a D:N ratio of approximately 3.65. In the work of Minkowski comparison has been made of the blood sugar content of dogs both in phlorhizin and pancreatic diabetes after kidney removal. He found that in pancreatic diabetes hyperglycaemia was in evidence whereas the blood sugar content rose little, if at all, above the normal in phlorhizin diabetes. It appears to the writer, however, that the conditions existing in the two experimental states were too dissimilar for strict comparison. In pancreatic diabetes a *constant* influence is at work for one may reasonably assume that extirpation of the pancreas either initiates a stimulus for, or removes an inhibition from, the sugar regulating mechanism. In phlorhizin diabetes, on the other hand, to produce a similar constant response on the part of the body the animal organism must be continually supplied with the necessary quantity of the drug. Expressed in other words, previous experiments have been per-

⁵ Pavy, Brodie and Siau: *Journ. of Physiol.*, xxix, p. 467, 1903.

formed with animals in unlike states; in pancreatic diabetes, a constant stimulus has been present, while little or no attempt has been made to imitate comparable conditions in phlorhizin diabetes. The production and maintenance of a D:N ratio of 3.65 would appear to satisfy the condition of a constant stimulus. Ablation or ligation of the kidneys *under these circumstances* would perhaps constitute an experiment with conditions more strictly comparable with those obtaining in pancreatic diabetes than is true for many of the previous investigations. Hitherto, for the most part, little or no attention has been devoted to the question of phlorhizin dosage or to the period of time which phlorhizin might reasonably be expected to exert an influence upon the percentage of sugar in the blood. At present it is well recognized that the glycosuric influence of a single injection of phlorhizin persists, in dogs at least, for a few hours only. Yet there are records of experiments of the type under discussion demonstrating the possibility that sufficient time had elapsed since the last phlorhizin injection for restoration of blood sugar content by utilization of an excess in the blood, provided a high percentage of sugar in the blood had been temporarily established.

The object of the present investigation has been a study of the changes in blood sugar content of animals in phlorhizin diabetes after ligation of the kidneys or suppression of the renal secretory function. Two types of experiments were planned. In the one, dogs have been brought into a condition of *total* diabetes with phlorhizin. The kidneys were then ligatured twice, one ligature being placed around the ureters and the blood vessels, another designed to include any collateral vascular branches. The experiments were so planned that the operation was performed upon the kidneys shortly after the morning administration of phlorhizin, and a second injection of the drug was given as usual eight hours subsequent to the first. Blood was analyzed for total solids and dextrose content just previous to the operation and at intervals subsequent to ligation of the renal organs.

In the other type of experiment the function of the kidney as an excretory organ was practically abolished in fasting phlorhizinized rabbits by the subcutaneous administration of sodium tartrate.*

* Cf. Underhill: this *Journal*, xii, p. 115, 1912.

In this instance the blood supply to the kidneys was presumably uninterrupted whereas the escape of sugar from the blood was prevented by the changed character of the tubular epithelium. The establishment of such a condition has been found to occur after tartrate injections and has been recorded in a previous paper. Blood analyses were made only after removal of the kidney function; for experience relative to the blood sugar content of both normal fasting rabbits and phlorhizinized rabbits made unessential the preliminary determination of the blood sugar content. By omission of the estimation of blood sugar content before abolition of the kidney function the well-known influence of the removal of a relatively large quantity of blood upon the percentage of sugar in the blood was obviated.

The double ligation of the kidneys in dogs practically amounted to extirpation of these organs and hence was equivalent to the non-participation of the kidneys in the sequence of phenomena following the operation. In the case of rabbits, however, the circulation through the kidneys was presumably more or less intact although the secreting mechanism was abolished.⁷ Any influence exerted by phlorhizin upon the renal organs in the last mentioned instance, such as *production* of sugar, would therefore be possible through the channel of the circulation. If the kidney is specifically responsible for the blood sugar phenomena exhibited in phlorhizin diabetes the results obtained from examination of the blood sugar content under the two conditions just outlined should theoretically at least be totally unlike. Ligation of the kidneys might be expected under the experimental conditions to maintain the condition of hypoglycaemia or at most to allow blood sugar content to become normal. If the kidneys actually *produce* sugar from some antecedent in the blood, as suggested by Pavy, the blood sugar content might be assumed to increase even above the normal by reabsorption in the absence of free secretion, provided it is granted that in the nephritis induced sufficient normal renal cells are present to accomplish such a task. On the contrary, if the renal cells after tartrate injection are totally incapacitated from producing sugar under the influence of phlorhizin, that is, practically every cell has lost its function—a condition which is most

⁷ Underhill: *loc. cit.*

unlikely—then the blood sugar should behave in the manner indicated for ligation of the kidney, that is, the sugar in the blood should not increase above the normal. It has been found that in general *with both types of experiments blood sugar content rose above the normal.*

For the establishment of hyperglycaemia under either of the two methods outlined, various explanations may be offered. In the first place it may be assumed that phlorhizin acts specifically upon the kidney rendering this organ more permeable for sugar, as suggested by v. Mering, and in its attempt to maintain blood sugar content normal the blood sugar regulating mechanism is thrown somewhat out of adjustment, the inhibition is removed or in a manner similar to antibody production there is a compensatory hyperfunction and, in the event of the removal of the kidney function, sugar increases in the blood until hyperglycaemia obtains. In the case of rabbits, from the standpoint of Pavy's suggestion, hyperglycaemia could be induced by *production* of sugar in the kidney and reabsorption into the blood. Finally, hyperglycaemia may be explained equally well on the assumption that phlorhizin has a two-fold action: (a) an influence upon the kidney, resulting in augmented permeability for blood sugar and (b) a *specific* activity upon some other mechanism whereby the organism continually produces new sugar which it throws into the blood stream. The latter action might be, however, less pronounced than the former; hence, under ordinary conditions hypoglycaemia is found associated with phlorhizin diabetes. The evidence adduced below points in this direction.

METHODS: Throughout this investigation the experimental animals were maintained in a state of inanition but water was freely given. With dogs total phlorhizin diabetes was established according to the procedure recommended by Lusk.⁸ For the operations, which were performed under aseptic conditions, anaesthesia was produced by ether only. After ligation of the kidneys no anaesthetic was necessary for withdrawal of blood samples from a femoral artery. Post-mortem examinations demonstrated in each case the complete ligation of the kidneys. No attempt was made in the experiments with rabbits to establish a fixed D:N

⁸ Lusk: *Amer. Journ. of Physiol.*, xxii. p. 163, 1908.

ratio, the animals receiving an injection of 0.25 gram phlorhizin subcutaneously once daily. Tartrate injections were also given subcutaneously. Blood sugar was estimated by the method commonly employed in this laboratory.⁹ Total solids of the blood were determined in the usual manner. Glycogen in the liver was estimated according to the procedure of Pflüger.¹⁰

The influence of ligation of the kidneys upon the blood sugar content of dogs in phlorhizin diabetes.

From the data presented in tables 1 and 2 several points of interest are indicated. In the first place it is apparent that the D:N ratio of 3.65 for fasting dogs established by Lusk was readily reached and that in this condition hypoglycaemia is in evidence.

TABLE 1.

Experiment 7, Dog X. Bitch of 9.5 kilos received three times daily subcutaneous injection of 2 grams phlorhizin.

DATE 1911	URINE		BLOOD		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	
	cc.	grams	grams	per cent per cent	
December					Urine was not collected until second day of phlorhizin administration.
19	600	9.45	36.60		D : N = 3.76.
20	620	10.20	36.52		D : N = 3.58.
21				17.75 0.062	Blood drawn just before ligation of kidneys.
					12.00 m. Kidneys ligated four hours after first daily phlorhizin injection.
					3.00 p.m. 2 grams phlorhizin as usual.
				15.30 0.172	6.00 p.m. Six hours after ligation of kidneys.
					8.00 p.m. Animal dead. The liver was glycogen free.

⁹ Underhill: this *Journal*, i, p. 113, 1905-06.

¹⁰ Pflüger: *Arch. f. d. ges. Physiol.*, cxi, p. 307, 1906.

The most noteworthy result of the experiments, however, is shown by the change in the blood sugar content of the blood after ligation of the kidneys. It will be observed in both experiments that *after ligation of the kidneys in dogs maintained in total phlorhizin diabetes the percentage of sugar in the blood rises above the normal*, although the hyperglycaemia was much more marked in one case than in the other. Accompanying this hyperglycaemia is a significant diminution in the percentage of total solids of the blood or, viewed from the opposite standpoint, an increase in the water content. If this fact is taken into consideration one may reasonably assume that the blood sugar content in relation to the other solids was actually increased to an extent even greater than the percentage figures indicate.

Comparison of these figures for blood sugar content with those for instance of Minkowski obtained in pancreatic diabetes after

TABLE 2.

Experiment 8, Dog Y. Bitch of 11.5 kilos received three times daily subcutaneous injection of 2 grams phlorhizin.

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	Dextrose	
	cc.	grams	grams	per cent	per cent	
December						
19	560	10.53	40.00			Urine was not collected until second day of phlorhizin administration.
20	610	10.14	35.87			D : N = 3.79.
21				22.45	0.087	D : N = 3.53.
						Blood drawn just before kidney ligation.
						10.30 a.m. Kidneys ligated 2.5 hours after first daily phlorhizin injection.
						3.00 p.m. 2.0 grams phlorhizin as usual.
				21.70	0.276	5.00 p.m. 7.5 hours after ligation of kidneys.
				21.70	0.306	11.00 p.m. 12.5 hours after ligation of kidneys.
						Animal bled to death. The liver was glycogen-free.

kidney extirpation, reveals the interesting fact that in both instances the type of response to the given stimulus is similar; for in each case there is a hyperglycaemia. It must be admitted, however, that in pancreatic diabetes the degree of hyperglycaemia is much more marked than in the condition induced by phlorhizin. It is also of significance that in our experiments the liver failed to reveal a trace of glycogen in the one hundred grams of tissue employed for analysis.

The results of these experiments justify the suggestion that in phlorhizin diabetes there may be two types of action. In the first place, there is exerted an influence upon the kidney whereby this organ becomes more permeable for blood sugar and secondly, there is evidence of the stimulation of another structure or mechanism that functions by producing sugar or perhaps by diminishing sugar destruction.

The behavior of the blood sugar content in phlorhizinized rabbits after suppression of kidney secretion by means of sodium tartrate.

In tables 3 to 8 inclusive may be found data concerning the percentage of sugar in the blood of phlorhizinized rabbits after suppression of kidney secretion by means of subcutaneous injections of sodium tartrate. Inspection of these data will clearly demonstrate an increased blood sugar content in most of the experiments after exclusion of the kidney secretion even though these animals were not in a condition of total diabetes. It is apparent therefore that in the rabbit, at least, a condition of total diabetes is not essential for the production of hyperglycaemia after suppression of the kidney function.

The observations recorded here do not exclude the possibility of the production and reabsorption of sugar by the kidneys as outlined by Pavy. However, in view of the fact that the type of response in this case coincides exactly with that observed for dogs where the kidneys were practically removed, it is probable that the mechanism in the two cases is similar for there is no obvious reason to assume that the character of reaction is different in the two species of animals. If our conception is correct, then it is obvious that sugar production and reabsorption by the kidney can play only an insignificant rôle at most, since in the case of

TABLE 3.

Experiment 1; Rabbit G. Female rabbit of 2400 grams received daily subcutaneous injection of 0.25 gram phlorhizin.

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	Dextrose	
	cc.	grams	grams	per cent	per cent	
November						
29	105	0.86	2.0			Would not drink water.
30	60	1.04	1.49			Would not drink water.
December						
1	85	1.36	0.98			Animal drank 80 cc. water.
2	32	0.072	0.072			Subcutaneous injection of 1.0 gram tartaric acid, neutralized with Na_2CO_3 , dissolved in 20 cc. water.
						Drank 60 cc. water.
3	3	0.007	trace			Drank 30 cc. water.
4	0			18.11	0.12	Blood drawn 5 hours after last phlorhizin injection. The liver was glycogen free.

TABLE 4.

Experiment 2, Rabbit H. Male rabbit of 2500 grams received daily subcutaneous injections of 0.25 gram phlorhizin.

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	Dextrose	
	cc.	grams	grams	per cent	per cent	
November						
29	100	0.56	2.30			No water intake.
30	90	1.31	2.57			No water intake.
December						
1	110	1.26	1.73			Water intake = 100 cc.
2	5	0.018	0.015			Subcutaneous injection of 1.5 grams tartaric acid neutralized with Na_2CO_3 , dissolved in 30 cc. water.
3	30	0.066	0.068			Water intake = 60 cc.
4	20		trace	16.61	0.18	Blood drawn 5 hours after last phlorhizin injection. The liver contained 0.35 gram glycogen.

TABLE 5.

Experiment 3, Rabbit I. Female rabbit of 2300 grams received daily subcutaneous injection of 0.25 gram phlorhizin.

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitro- gen	Dex- trose	Total Solids	Dex- trose	
	cc.	grams	grams	per cent	per cent	
December						
5	100	2.88	4.03			Water intake = 40 cc.
6	200	1.73	3.45			Water intake = 125 cc.
7	90	1.84	1.40			Water intake = 50 cc.
8	10	0.075	0.076			Subcutaneous injection of 2.0 grams tartaric acid, neu- tralized with Na_2CO_3 , dis- solved in 30 cc. water.
9	0			18.0	0.21	Water intake = 150 cc. Blood drawn 2.5 hours after last phlorhizin in- jection. The liver con- tained 0.41 gram glyco- gen.

TABLE 6.

Experiment 4, Rabbit J. Female rabbit of 2800 grams received daily subcutaneous injection of 0.25 gram phlorhizin.

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitro- gen	Dex- trose	Total Solids	Dex- trose	
	cc.	grams	grams	per cent	per cent	
December						
5	250	1.45	2.54			Water intake = 170 cc.
6	175	2.70	4.46			Water intake = 80 cc.
7	125	2.38	2.75			Water intake = 90 cc.
8	10	0.075	0.076			Subcutaneous injection of 2.0 grams tartaric acid, neu- tralized with Na_2CO_3 , dis- solved in 30 cc. water.
9	0			18.70	0.15	Water intake = 240 cc. Blood drawn 2.5 hours after last phlorhizin in- jection. The liver con- tained 0.60 gram glyco- gen.

dogs the blood sugar content increased above the normal after practical extirpation of the kidneys. It is difficult to comprehend how extensive kidney secretory activity could be alleged in the rabbit experiments carried through in the manner described. It is evident therefore that under the experimental conditions here outlined little support can be derived in favor of the view advanced by Pavy. On the other hand, in view of the new facts furnished by the present investigation the conception of v. Mering with respect to the nature of the mechanism of phlorhizin diabetes has been supplemented and extended.

TABLE 7.

Experiment 5, Rabbit K. Female rabbit of 2800 grams received daily subcutaneous injection phlorhizin.

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	Dextrose	
	cc.	grams	grams	per cent	per cent	
December 11	85	1.13	2.40			Water intake = 25 cc. Injected 0.25 gram phlorhizin.
12	100	1.35	1.58			Water intake = 70 cc. Injected 0.25 gm. phlorhizin.
13	10	0.015	0.008			Subcutaneous injection of 2.0 grams tartaric acid, neutralized with Na_2CO_3 , dissolved in 15 cc. water. INJECTED 1.0 GRAM PHLORHIZIN. Water intake = 195 cc.
14	0			18.65	0.20	Blood drawn 3.5 hours after injection of 1.0 gram phlorhizin. The liver was glycogen free.

TABLE 8.

Experiment 6, Rabbit L. Female rabbit of 2800 grams received daily subcutaneous injection of phlorhizin.

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitro- gen	Dex- trose	Total Solids	Dex- trose	
	cc.	grams	grams	per-cent	per cent	
December						
11	120	1.71	3.30			Water intake = 50 cc.
12	120	2.21	2.01			Water intake = 50 cc.
13	2	0	0			Subcutaneous injection of 3.0 grams tartaric acid, neutralized with Na_2CO_3 , dissolved in 20 cc. water. INJECTED 1.0 GRAM PHLORHIZIN. Water intake = 150 cc.
14	0			16.70	0.25	Blood drawn 4 hours after injection of 1.0 gram phlorhizin. The liver was glycogen free.

SUMMARY.

The mechanism of phlorhizin diabetes has been subjected to investigation after the removal of the renal secretory function by (a) ligation of the renal structures in the dog and (b) abolition of kidney secretion through subcutaneous administration of sodium tartrate to rabbits.

In both conditions a significant hyperglycaemia may be in evidence. With dogs this is accompanied by a decrease in the proportion of solids in the blood, that is, the water content is increased.

The data presented lead to the suggestion that phlorhizin may possess a two-fold action (a) an influence is exerted upon the kidney whereby this organ becomes more permeable for blood sugar and (b) an action upon other structures resulting in the production of sugar in quantities sufficient to cause hyperglycaemia if the kidney function is removed.

ANIMAL CALORIMETRY.

THIRD PAPER.

METABOLISM AFTER THE INGESTION OF DEXTROSE AND FAT, INCLUDING THE BEHAVIOR OF WATER, UREA AND SODIUM CHLORIDE SOLUTIONS.¹

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I. INTRODUCTION.

The discussion in the last paper of this series noted the fact that, whereas Magnus-Levy found an increase of 20 per cent in the metabolism of a dog during the hours following carbohydrate ingestion, Rubner's work showed an increase of but 5.8 per cent

¹ The urinary analyses of Dog II were accomplished by Miss Mary B. Wishart.

during a twenty-four-hour period following the ingestion of cane-sugar in such quantity as to furnish energy sufficient to provide for the daily necessities.

Heilner² repeated Rubner's experiments, using dextrose instead of cane-sugar and found an increase in the heat production equal to 4 per cent in twenty-four hours. The animal here employed was kept, as in Rubner's experiments, at an environmental temperature of 33°. This work seemed to justify Rubner's conclusion that carbohydrate is a material which may simply replace an isodynamic quantity of fat in metabolism, without materially altering the heat production.

Zuntz³ has criticised Heilner's results on the ground that the respiratory quotient was not determined, and therefore the true conditions were not accurately portrayed. In Zuntz's opinion the metabolism was really higher than Heilner calculated.

The respiration calorimeter has been used in the following series of experiments to throw additional light upon this problem.

II. EXPERIMENTAL PART.

Experiments were made upon Dog I but still more extensive work was accomplished with Dog II. The dogs were both kept at an environmental temperature of between 26° and 27° when within the calorimeter. The urine was always free from sugar. Other details of the procedure are given in the last paper.

A. Results of the ingestion of dextrose by Dog I.

In the previous paper it was demonstrated that the minimal basal metabolism of Dog I during a morning hour the day after meat had been given at noon was 22.3 calories.

Table I of the Appendix presents the metabolism of this dog during the second, third, fourth and fifth hours after the ingestion of 103 grams of dextrose which was taken with 400 cc. of water. Twenty-four hours before this, the dog had received 200 grams of rice. The calculated metabolism rose from a base level of 89 calories for four hours to 115, a difference of 26 calories or 6.5 per

² Heilner: *Zeitschr. f. Biol.*, 1, p. 488.

³ Zuntz: *Naturwissenschaftliche Rundschau*, xxi, No. 38, 1906.

hour. This represents an increase of 20 per cent. Since 103 grams of dextrose contain 396 calories and cause an increase in metabolism equal to 26 calories, it follows that 100 calories in dextrose would cause an increase of 6.6 calories. The non-protein respiratory quotients were 1.02, 0.99, 0.99 and 0.98 in the successive hours, indicating dominant carbohydrate oxidation.

Of 114.56 calories produced by the animal as calculated from the products of excretion and the oxygen absorption 110.05 calories were measured by the calorimeter. The difference is to be attributed to the greater proportional warming of the skin than of the rectal region, which, in the last paper, was shown to occur in this dog after the ingestion of 100 grams of dextrose. The urine of the period was free from sugar.

This experiment demonstrated without doubt a large rise in the heat production following the ingestion of dextrose by a dog. The respiratory quotients indicate that this increased metabolism could not have been due to the process of converting dextrose into fat.

This completes the record of work to be published with regard to Dog I.

B. The basal metabolism of Dog II.

A variety of problems were investigated upon Dog II. The animal was maintained in good condition and nearly constant weight from November 24, 1911 to May 3, 1912, upon a daily diet consisting of 100 grams of biscuit meal, 100 grams of chopped beef heart meat, 20 grams of fat and 10 grams of bone ash, containing together 5.25 grams of nitrogen and 700 calories. The diet was mixed with water and given to the animal at 6.00 p.m. The *basal metabolism* was determined on the day after the food ingestion usually between the hours of 1.00 p.m. and 4.00 p.m. If the action of a substance were to be determined, it was fed to the dog at noon and the metabolism determined from 1.00 p.m. to 4.00 p.m. or later. If 50 grams of sugar were given at this time, 50 grams of biscuit meal were deducted from the evening ration.

The basal metabolism was found to be the following on the various dates mentioned.

DATE	EXPERIMENT NUMBER	CALORIES		
		Calculated	Found	Average per hour (calculated)
December 2, 1911.....	3	17.53	17.61	17.53
January 9, 1912.....	10	15.45	14.54	15.45
January 22, 1912.....	13	15.41	14.47	15.20
		14.99	17.18	
		30.40	31.65	
January 26, 1912.....	17	15.73	15.89	16.16
		17.44	16.40	
		15.32	15.42	
		48.49	47.71	
January 30, 1912.....	18	16.65	14.22	16.86
		15.99	14.98	
		17.96	16.99	
		50.60	46.19	
February 5, 1912.....	21	15.43	15.11	16.56
		16.03	16.37	
		18.33	17.35	
		49.79	48.83	
April 22, 1912.....	51	14.67	16.10	15.56
		15.22	15.51	
		16.78	17.26	
		46.67	48.87	
Average.....				16.19

The average heat production of this basal metabolism is 16.2 calories. When any three-hour period is compared with this fundamental figure, the average of the three hours is always within 0.7 of a calorie or 4 per cent of the above figure. A value which comes between 15.5 and 16.9 calories may therefore be considered as within the limits of the normal basal value of this dog during sleep. The full details of these experiments are presented in the Appendix, Table II.

Assuming a minimal basal metabolism of 16.2 calories per hour the total would be 388.8 calories in twenty-four hours for a dog weighing 9.3 kgms. The area of the dog is 0.4956 square meters calculated from the formula, $11.2 \sqrt[3]{9.3^2}$. The minimal metabolism would therefore be 784 calories per square meter of surface, which may be contrasted with 759 calories similarly determined for Dog I. The difference is 3 per cent.

C. The results of the ingestion of dextrose by Dog II.

a. Twenty grams of dextrose. After giving 20 grams of dextrose in 150 cc. of water to Dog II at noon, no definite rise in metabolism could be made out in one experiment (No. 39) while in a second experiment (No. 42) the metabolism rose from the basal value of 16.2 to 18.7 calories in the second hour after the ingestion of the solution. The metabolism returned in the third hour to the basal value. This rise of 2.5 calories is an increase of 15 per cent above the basal metabolism. Since 20 grams of dextrose containing 75 calories causes a rise in heat production of 2.5 calories, it may be estimated that 100 calories would bring about an increase of 3.3 calories.

The essential points are here presented and the full details will be found in Table III of the Appendix.

EXPERIMENT NUMBER	TIME	NON-PROTEIN R. Q.	CALORIES	
			Calculated	Found
39	1.00-2.00 p.m.	1.12	16.94	19.12
	2.00-3.00	0.95	16.89	17.24
	3.00-4.00	0.88	15.75	16.55
			49.58	52.92
42	1.00-2.00 p.m.	1.08	18.72	18.60
	2.00-3.00	0.97	15.43	16.09
			34.15	34.69

It is difficult to explain the high non-protein respiratory quotients. It is apparent (Experiment 42) that a small quantity of

dextrose may affect the metabolism during the second hour after its ingestion and then the metabolism reverts to the basal value. It is also true that the ingestion of a small amount of dextrose may not increase the metabolism. It has been pointed out by Johannsson⁴ that after giving a small quantity of dextrose 75 grams to a fasting man there is no change in the metabolism on account of the retention of dextrose in the organism in the form of glycogen.

b. Fifty grams of dextrose. The ingestion of 50 grams of dextrose dissolved in 150 cc. of water caused a rise in the metabolism from the basal level of 16.2 calories to 19.6 in the second hour, which level was very nearly maintained during the third and fourth hours and then fell to the basal value in the fifth hour.

From the results of four experiments given in detail in Table II of the Appendix, the following type may be taken in illustration.

STRENGTH OF P. NUMBER	TIME	WGT. PERCENT R. Q.	CALORIES	
			Calculated	Found
29	1 (H) 2 (H) p.m.	0.96	19.59	19.94
	2 (H) 3 (H)	0.96	19.47	18.45
12	3 (H) 4 (H)	1.03	18.23	19.26
	4 (H) 5 (H)	0.83	16.61	17.25
			73.90	74.86

It is apparent, from the non-protein respiratory quotients of 98, 96 and 103, that carbohydrate combustion dominated during the second to fourth hours. In the fifth hour the quotient fell.

The rise from the basal value of 16.2 to 19.6 calories represented an increase of 3.4 calories or 20 per cent. The total increase in metabolism during the four hours was 9.1 calories. Since 50 grams of dextrose contain 184.6 calories, it follows that, taken in Rubner's sense, the ingestion of 100 calories of dextrose causes an increased metabolism amounting to 4.9 calories.

c. Seventy-five grams of dextrose. Administration of 75 grams of dextrose in 200 cc. of water caused an initial rise in metabolism from 16.2 to 19.6 calories, the same as produced by 50 grams of dextrose. The increased metabolism was, however, continued

⁴ Johannsson: *Skand. Arch. f. Physiol.*, xxi, p. 1, 1909.

at least through the fifth hour at a level of about 20 calories per hour. The total increase during the four hours was 16.35 calories. Since 75 grams of dextrose contain 277 calories, it may be calculated that 100 calories of dextrose cause an increase in metabolism equal to 5.9 calories.

The following table illustrates these facts, which are set forth in greater detail in the Appendix, Table II.

EXPERIMENT NUMBER	TIME	NON-PROTEIN R. Q.	CALORIES	
			Calculated	Found
40	1.00-2.00 p.m.	1.05	19.60	20.85
	2.00-3.00	1.01	20.22	21.28
	3.00-4.00	0.98	21.09	20.38
	4.00-5.00	1.04	20.24	19.44
			81.15	81.95

The respiratory quotient is such as to indicate oxidation of dextrose throughout the four hours. Although the results here presented do not justify the assumption that the higher metabolism terminated with the fifth hour after dextrose ingestion, yet there is indirect evidence which will be offered in the next paper of this series that this is so. This evidence consists in the fact that after giving 50 grams of dextrose, a large excretion of water by the kidney marks the fourth hour, the last hour of increased metabolism, whereas after giving 75 grams, the large excretion of water marks the fifth hour.

d. The nitrogen elimination after dextrose ingestion. In the calculations made above, the nitrogen of the urine was collected for the whole period beginning with the catheterization which took place just before placing the animal in the calorimeter and ending with the catheterization after removal of the dog from the box at the end of the experiment, and this nitrogen was apportioned equally between the hourly periods. As a matter of fact, however, the nitrogen elimination is far from even after the administration of 50 grams of dextrose, as will be seen from the following analyses:

Urinary analyses after giving 50 grams of dextrose at noon.

DATE	TIME	TOTAL N	NH ₄ -N	UREA- N	DATE	TOTAL N
		gram	gram	gram		gram
January 12-'12	11.00-12.00				January 16-'12	0.137
	12.00- 1.00	0.138	0.011	0.101		0.124
	1.00- 2.00	0.166	0.012	0.133		0.154
	2.00- 3.00	0.180	0.018	0.156		0.190
	3.00- 4.00	0.264	0.014	0.225		0.181
	4.00- 5.00	0.181	0.011	0.163		0.107

It was incidentally noticed that the volume of urine obtained between 3.00 and 4.00 o'clock was very large in quantity, as compared with all the other hours, and often reached 100 cc. This led to the work to be described in the next paper. This hour in question was also characterized by a large nitrogen excretion. The first hour showed a diminished excretion of nitrogen and a small excretion of water. Under these circumstances, it was concluded that the hourly excretion of nitrogen could not be a measure of the protein metabolism of the period but rather an indication of a greater or less secretion of urea through the kidney. It was, therefore, deemed nearest correct to evenly distribute through the different hours the total nitrogen elimination for the whole experimental period while the dog was in the calorimeter, in order to obtain a measure of the protein metabolism.

D. Discussion of the results of dextrose ingestion.

Some of the results of the work on metabolism after dextrose ingestion may be seen in the following table.

	DEXTROSE	RISE IN METABOLISM IN SECOND HOUR	DURATION OF INCREASED METABOLISM	TOTAL INCREASE IN METABOLISM	100 CALORIES DEXTROSE INCREASES METABOLISM
	grams	per cent	hours	calories	calories
Dog I.....	103	20	5*	26.00	6.6
Dog II.....	20	15	2	2.50	3.3
	50	20	4	9.10	4.9
	75	20	5*	16.35	5.9

*At least. See discussion p. 33.

Here appears a general confirmation of the work of good authorities. The increase in metabolism of 20 per cent found by Magnus-Levy is verified, and a "specific dynamic" action of dextrose of 4.9 after giving 50 grams of dextrose is in accord with Rubner's experiments with cane-sugar. Rubner's theory was that the extra heat produced by cane-sugar is derived from a cleavage of cane-sugar into levulose and dextrose. His idea calls for the absorption of dextrose and its utilization in isodynamic replacement of fat in the metabolism. However, this does not occur. *During the first four and five hours after the ingestion of 50, 75 and 100 grams of dextrose, the heat production rises 20 per cent above the basal metabolism.* This fact was lost to sight in experiments continued over a period of twenty-four hours.

What then is the cause of the increased metabolism? Dextrose itself is soluble, ready for absorption and ready for oxidation by the cells. Johansson has already been quoted as having shown the absorption of 75 grams of dextrose by a fasting man who retained it as glycogen and no increase in metabolism followed. The same writer⁵ has given to a diabetic 50 grams of dextrose which was excreted unchanged in the urine and caused no change in the metabolism. The question of "Darmarbeit" may, therefore, be excluded as a factor to be considered.

Two causes might contribute to the increased metabolism.

1. The sugar entering the blood stream might set up osmotic changes which would stimulate the cells to increased movement and, therefore, increase the metabolism.
2. The larger quantity of dextrose brought by the blood might cause increased oxidation through the presence of an increased amount of a readily oxidizable food-stuff.

To investigate the first problem it was decided to test the metabolism of the dog by giving: (1) 200 cc. of water; (2) 150 cc. of water containing 6.7 grams of sodium chloride; (3) 150 cc. of water containing 17 grams of urea. The solutions of salt (assuming complete electrolytic dissociation) and of urea were isotonic with 50 grams of dextrose in 150 cc. of water.

To test the second problem another food-stuff, olive oil in fine emulsion, was given to the dog.

⁵ Johansson: *loc. cit.*

E. The ingestion of water.

That water in moderate quantities has no effect on metabolism has already been set forth by Bidder and Schmidt,⁶ by Rubner⁷ and by Heilner.⁸ The following results were obtained after giving 200 cc. of water at noon (Appendix, Table I) and also, for comparison, the results of the day following when no food was given at noon (Appendix, Table II). The dog was given the regular standard diet at 6.00 p.m. as in all other experiments.

*Experiment 16—200 cc. of water,
January 25, '12.*

*Experiment 17—No food, January
26, '12.*

TIME	CALORIES		TIME	CALORIES	
	Calculated	Found		Calculated	Found
1.00-2.00 p.m.	16.52	17.22	1.00-2.00 p.m.	15.73	15.89
2.00-3.00	16.52	17.69	2.00-3.00	17.44	16.40
3.00-4.00	16.46	17.34	3.00-4.00	15.32	15.42
	49.50	52.25		47.71	48.49

It is evident that the ingestion of that quantity of water in which the dextrose given in the experiments was dissolved, can have no influence on the metabolism.

F. The ingestion of urea in solution.

In Experiment 14, 17 grams of urea in 150 cc. of water were given the dog at noon and the animal was placed in the calorimeter. At 12.45 this was largely vomited. The experiment was continued for two hours, although the measurement by the calorimeter during the first hour is not to be relied upon, on account of the mass of water on the floor of the box.

In Experiment 19, 12 grams of urea were given, which was properly retained by the animal. The results of these experiments are presented below (full details, Appendix, Table II).

⁶ Bidder and Schmidt: *Verdaugungssäfte und Stoffwechsel*, 1852, p. 340.

⁷ Rubner: *Gesetze des Energieverbrauchs*, 1902, p. 62.

⁸ Heilner: *Zeitschr. f. Biol.*, xlix, p. 373, 1907.

*Experiment 14—17 grams (?) urea,
150 cc. water, at noon.*

*Experiment 19—12 grams
urea, 150 cc. water, at noon.*

TIME	CALORIES		CALORIES	
	Calculated	Found	Calculated	Found
1.00-2.00 p.m.	15.85	20.42 (?)		
2.00-3.00	17.76	18.76	16.35	15.94
3.00-4.00			16.68	16.07
	33.61		33.03	32.01

In Experiment 14, during the period before the dog was put into the calorimeter, the nitrogen eliminated amounted to 0.154 gram hourly, whereas, after administration of the urea solution, it rose to 0.424 gram per hour, indicating a considerable absorption of urea. In the successful Experiment 19, the hourly excretion of nitrogen rose from 0.133 gram before the ingestion of urea to 0.602 gram after, which corresponds to the elimination of an extra gram of urea per hour. As the results show, *the elimination of urea after its administration in solution is without any influence upon metabolism.* The normal of 16.2 calories per hour was closely approximated.

Zuntz⁹ has sought to attribute part of the "specific dynamic" action of protein to the increased kidney activity accompanying the elimination of urea, but the above experiment shows this to be without foundation.

The calculation of the protein metabolism in Experiment 14 and Experiment 19 was on the assumption that it was maintained at the same height as on near-by days when no food was given during the same interval of the day.

G. The ingestion of sodium chloride in solution.

One experiment was made to determine the influence of the ingestion of 6.7 grams of sodium chloride in 150 cc. of water, a solution which is isotonic with 50 grams of dextrose in 150 cc. of water. This was given at noon on two successive days, the metabolism being obtained on the second day with the following results (details, Appendix, Table II).

⁹ Zuntz: *Zentralbl. f. Physiol.*, xxiii, p. 960, 1909.

Experiment 15—4" grams NaCl in 150 cc. of water.

TIME	CALORIES	
	Calculated	Found
1 (0)-2 (0) p.m.	15.09	15.00
2 (0)-3 (0)	14.64	17.37
3 (0)-4 (0)	14.19	16.54
	43.92	51.91

These figures contradict the statement of Zuntz¹⁹ regarding the behavior of sodium chloride on metabolism. The results in no way differ from the average basal metabolism of 16.2 calories per hour. Hence, the ingestion of 150 cc. of a solution containing 4.2 per cent sodium chloride is without influence upon the metabolism.

The urinary excretion of sodium chloride during the experimental period between 12.00 noon and 4.08 p.m. amounted to 0.805 gram per hour and from 4.08 to 10 a.m. the next day to 0.439 gram per hour. The quantity of urine was 140 cc. during the experimental period of four hours, indicating marked diuresis.

H. The ingestion of fat.

Since dextrose solutions caused an increased metabolism and solutions such as 8 per cent urea and 4.2 per cent sodium chloride and water itself had no effect, it seemed desirable to test the influence of another food-stuff, fat, which is quickly absorbed, enters the blood current and supplies the cells with nutriment.

Fifty grams of olive oil were mixed with 10 cc. of a 1.2 per cent sodium carbonate solution and shaken till a fine emulsion formed, and this was given to the dog at noon.

The results may be thus summarized (details, Appendix, Table II).

Experiment 23—50 grams olive oil, at noon.			Experiment 24—50 grams, olive oil, at noon.	
TIME	CALORIES		CALORIES	
	Calculated*	Found	Calculated	Found
1 (0)-2 (0) p.m.		18.71	17.69	19.77
2 (0)-3 (0)		18.05	19.71	19.73
		36.76	37.40	39.50

*Oxygen lost, but the CO₂ per hour was 6.05 and 5.91 against 5.81 in experiment 24, on day following, during which hour the calculated metabolism was 17.69, the R. Q. being 0.79. Given these latter conditions, the calculated metabolism of experiment 23 would have been 18 calories and over in the two hours recorded.

¹⁹ Zuntz: *Zentralbl. f. Physiol.*, xxiii, p. 960, 1909.

These experiments illustrate an increased metabolism above the basal level of 16.2 calories per hour, due to the inflow of fat. If one again recalls Benedict's experiments showing the negative effect of cathartics and of agar-agar upon the metabolism, one cannot attribute this increase to "Darmarbeit." The emulsified fat flows partly through the portal vein, but in large quantity enters directly into the circulation through the thoracic duct, at once affording a means of enrichment of the fat content of the general circulation. Under these circumstances of ample nutrition, the metabolism increases (in the sense of Voit).

The general results obtained from the experiments mentioned in this paper are shown in the accompanying chart.

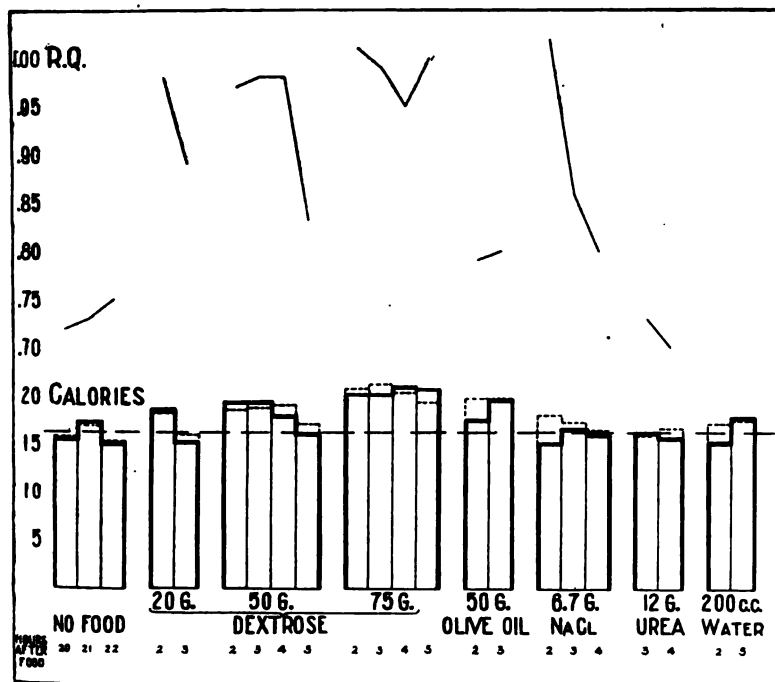


CHART 1 ILLUSTRATING THE EFFECT OF THE INGESTION OF DEXTROSE AND FAT AND OF WATER, UREA AND SALT SOLUTIONS ON THE METABOLISM.

Solid lines—metabolism in calories as calculated. Broken lines—metabolism in calories as found.

III. SUMMARY.

It is impossible to discuss the present work without anticipatory reference to the results of Miss Fisher and Miss Wishart, which are to be presented in the paper which follows this. These authors have found that after giving 50 grams of dextrose to a dog, the blood sugar rises in percentage within one hour and then falls to normal during the succeeding hours; that there is a large retention of water by the organism during the period of high metabolism, which water is suddenly eliminated during the last hour of the high metabolism (the fourth after dextrose ingestion); that during this same hour, the absorption of dextrose is completed; and that, during the period of high metabolism and water retention, the percentage of hemoglobin falls in the blood, rising again after the water elimination.

In general, the following picture may be drawn. It was noted in Dog I, that, after giving 100 grams of dextrose, the skin temperature rose during the second hour, while the rectal temperature fell slightly (second paper). When 50 grams of dextrose are given (Dog II) the sugar content of the blood in per cent is first increased, but by the end of the second hour and thereafter, it is found to have returned to its normal percentage value. The volume of the blood, however, increases during the second hour, obviously in response to the osmotic pressure exerted by the increased sugar concentration. The increase in blood volume is demonstrated by the fall in the hemoglobin content of the blood. During this period, the metabolism shows an increase of 20 per cent above the normal basal value. During the fourth hour there is final absorption of all the dextrose ingested, the metabolism remains high and there is a large excretion of water in the urine. At the end of the same fourth hour, the blood sugar content is normal, indicating that, during the withdrawal of water and the concentration of the blood, the liver and muscles maintain the normal sugar content of the blood. During the second, third and fourth hours, the high metabolism is accompanied by respiratory quotients of about 1.00 (after allowing for the influence of protein metabolism). However, during the fifth hour, when the metabolism has returned to its basal value, a non-protein respiratory quotient of 0.83 indicates that a mixture of fat and carbohydrate is being oxidized. The glycogenic function apparently enters into control and reduces the amount of carbohydrate available for combustion, and fat is oxidized as well.

It is interesting to recall in this connection that Hári¹¹ found that the respiratory quotient was increased in a fasting animal after giving adrenalin. Adrenalin causes a discharge of sugar from the glycogen repositories of the organism,¹² the percentage of blood sugar rises largely and, in virtue of this, carbohydrate tends to replace fat in the oxidative processes.

If 20 grams of dextrose be given, a rise of 15 per cent may take place during the second hour after sugar ingestion, to be followed by a normal basal metabolism in subsequent hours.

If 75 grams of dextrose be given, a 20 per cent increase in metabolism continues from the second through the fifth hour, during which hour the volume of urine becomes large, indicating that this is the final hour of the higher metabolism.

It is shown that ingestion of 200 cc. of water, of 150 cc. of a 4.2 per cent solution of sodium chloride or of 150 cc. of an 8 per cent solution of urea have no influence upon metabolism. It is, therefore, concluded that the high metabolism after giving dextrose is not due to osmotic changes between the blood and the tissues. The only remaining conclusion which appears possible is that *the increase in metabolism is due to the presence of a greater amount of free diffusible carbohydrate than is present when there is no absorption of carbohydrate from the intestines.* It is known that the sugar of the blood behaves as if it existed in chemical combination (Loewi; Lépine). If this combining power over sugar remains constant, then when the blood is diluted, with coincident maintenance of the normal percentage content of dextrose, there would be an increased supply of free readily oxidizable sugar molecules available for the nutrition of the cells. Hence, carbohydrate alone is oxidized, and the metabolism rises in virtue of an increased supply of nutriment.

An emulsion of olive oil ingested by the dog caused an increase in metabolism, probably for similar reasons.

These experiments confirm the work of Zuntz and of Rubner in many particulars, but they bring to light new points which show that the theories of both Zuntz and Rubner will have to be revised.

The views here expressed, however, conform to the "older" view of Carl Voit, who believed that the presence of abundant food increased the power of the cells to metabolize.

¹¹ Hári: *Biochem. Zeitschr.*, xxxviii, p. 23, 1912.

¹² Ringer: *Journ. of Exp. Med.*, xii, p. 105, 1910.

TABLE I.

DATE	TIME	EXP. NO.	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN		
								CO ₂	O ₂	R. Q.
May 4, '11.....	10.00-11.00 a.m.	34	grams 10.85	grams 7.94	0.99	grams 9.27	grams 0.213	grams 8.86	grams 6.14	1.02
	11.00-12.00		10.68	8.15	0.95	9.06	0.213	8.65	6.35	0.99
	12.00-1.00 p.m.		11.30	8.59	0.96	9.28	0.213	9.28	6.79	0.99
	1.00-2.00		11.23	8.64	0.95	9.31	0.213	9.20	6.84	0.98

TABLE II.

DATE	TIME	EXP. NO.	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN		
								CO ₂	O ₂	R. Q.
December 2, '11	9.57-10.57 a.m.	3	grams 5.61	grams 5.21	0.76	grams 9.87	grams 0.133	grams 4.03	grams 4.09	0.72
January 9, '12	10.00-11.00 a.m.	10	5.40	4.72	0.83	9.41	0.1331	4.14	3.50	0.86
January 22, '12	1.00-2.00 p.m.	13	5.56	4.55	0.89	8.66	0.124	4.40	3.50	0.92
	2.00-3.00		5.26	4.47	0.86	8.25	0.124	4.11	3.42	0.87
January 26, '12	1.00-2.00 p.m.	17	4.75	4.83	0.72	7.31	0.095	3.89	4.03	0.70
	2.00-3.00		5.26	5.35	0.73	7.50	0.095	4.40	4.55	0.70
	3.00-4.00		4.84	4.67	0.75	7.23	0.095	3.96	3.87	0.74
January 30, '12	1.00-2.00 p.m.	18	5.14	5.12	0.73	8.60	0.130	3.92	4.02	0.71
	2.00-3.00		5.14	4.78	0.78	7.97	0.130	3.92	3.78	0.75
	3.00-4.00		5.53	5.52	0.73	8.03	0.130	4.33	4.42	0.71
February 5, '12	1.00-2.00 p.m.	21	5.32	4.60	0.84	6.51	0.094	4.44	3.80	0.85
	2.00-3.00		5.11	4.88	0.76	6.27	0.094	4.22	4.08	0.75
	3.00-4.00		5.61	5.61	0.73	6.54	0.094	4.73	4.81	0.72
April 22, '12...	12.00-1.00 p.m.	51	5.03	4.38	0.84	10.29	0.132	3.80	3.26	0.85
	1.00-2.00		4.98	4.60	0.79	8.99	0.132	3.75	3.48	0.78
	2.00-3.00		5.23	5.12	0.74	8.18	0.132	4.00	4.00	0.73

In experiments 3 and 10 standard diet was given at noon, in the others at 6 p. m. daily.

DOG I.

CALORIES				BODY TEMPERATURE			MORNING WEIGHT	FOOD
Protein	Non-Protein	Total Calculated	Total Found	Start	End	Difference		
5.64	21.70	27.34	26.04	37.82	38.15	+0.33	Kilograms 16.3	103 grams dextrose + 400 cc. water at 9.15 a.m.
5.64	22.42	28.06	26.42		38.19	+0.04		
5.64	24.03	29.67	26.22		38.08	-0.11		
5.64	23.85	29.49	31.37		38.45	+0.37		
		114.56	110.05					

DOG II.

CALORIES				BODY TEMPERATURE			MORNING WEIGHT	BEHAVIOR OF DOG	REMARKS
Protein	Non-Protein	Total Calculated	Total Found	Start	End	Difference			
4.07	13.46	17.53	17.61	37.89	37.85	-0.04	kg. 9.3	Very quiet.	No food.
3.51	11.94	15.45	14.54	38.44	38.44		8.9	Quiet.	No food.
3.29	12.12	15.41	14.47	38.66	38.45	-0.21	8.7	Very quiet.	No food.
3.29	11.70	14.99	17.18		38.48	+0.03		Active 7 minutes.	
		30.40	31.65					O ₂ low.	
2.52	13.21	15.73	15.89	38.36	38.26	-0.10	8.8	Very quiet.	No food.
2.52	14.92	17.44	16.40		38.27	+0.01		Slight movements.	
2.52	12.80	15.32	15.42		38.22	-0.05		Moving 1 minute.	
		48.49	47.71						
3.45	13.20	16.65	14.22	38.43	38.32	-0.11	8.8	Quiet.	No food.
3.45	12.54	15.99	14.98		38.28	-0.04		Occasional movement.	
3.45	14.51	17.96	16.99		38.43	+0.15		Active 1 minute.	
		50.60	46.19						
2.50	12.93	15.43	15.11	38.40	38.31	-0.09	9.0	Very quiet.	No food.
2.50	13.53	16.03	16.37		38.44	+0.13		Occasional movements.	
2.50	15.83	18.33	17.35		38.62	+0.18		Active 2 minutes.	
		49.79	48.83						
3.58	11.09	14.67	16.10	37.59	37.58	-0.01	9.4	Very quiet.	No food.
3.58	11.64	15.22	15.51		37.51	-0.07		Very quiet.	
3.58	13.20	16.78	17.26		37.64	+0.13		Quiet.	
		46.67	48.87						

TABLE II.

DATE	TIME	EXP. NO.	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN		
								CO ₂	O ₂	R. Q.
March 26, '12.	1.00-2.00 p.m.	39	grams	grams		grams	grams	grams	grams	
			6.98	4.87	1.04	7.11	0.150	5.58	3.60	1.11
	2.00-3.00		6.22	4.97	0.91	7.33	0.150	4.82	3.70	0.95
	3.00-4.00		5.49	4.72	0.84	7.04	0.150	4.09	3.45	0.86
March 30, '12.	1.00-2.00 p.m.	42	7.46	5.54	0.98	9.08	0.200	5.59	3.75	1.06
	2.00-3.00		5.74	4.68	0.89	8.63	0.200	3.87	2.89	0.97
January 3, '12.	10.20-11.20 a.m.	9	5.27	4.47	0.86	9.08	0.161	3.78	3.10	0.89
	1.00-2.00 p.m.		7.69	5.77	0.97	10.76	0.136	6.42	4.62	1.01
	2.00-3.00		8.34	6.76	0.90	10.89	0.136	7.04	5.61	0.91
	3.00-4.00		9.63	7.59	0.92	12.51	0.136	8.36	6.44	0.94
	4.00-5.00		6.27	5.02	0.91	10.30	0.136	4.99	3.87	0.94
January 12, '12.	1.00-2.00 p.m.	11	7.59	5.70	0.97	8.71	0.143	6.23	4.49	1.00
	2.00-3.00		7.65	5.69	0.98	8.28	0.143	6.31	4.48	1.01
January 18, '12.	3.00-4.00 p.m.	12	7.10	5.27	0.98	9.22	0.133	5.87	4.15	1.03
	4.00-5.00		5.71	4.99	0.83	8.62	0.133	4.44	3.87	0.83
February 2, '12.	1.00-2.00 p.m.	20	7.41	5.71	0.94	8.41	0.150	6.01	4.44	0.98
	2.00-3.00		7.25	5.70	0.93	8.49	0.150	5.87	4.43	0.96
March 27, '12.	1.00-2.00 p.m.	40	7.83	5.66	1.01	8.19	0.115	6.76	4.69	1.05
	2.00-3.00		7.82	5.79	0.99	7.91	0.115	6.75	4.82	1.01
	3.00-4.00		8.01	6.14	0.95	8.39	0.115	6.94	5.17	0.98
	4.00-5.00		8.07	5.84	1.00	7.99	0.115	7.00	4.87	1.04
January 25, '12.	1.00-2.00 p.m.	16	5.31	4.60	0.84	7.63	0.130	4.11	*3.50	0.85
	2.00-3.00		5.13	5.51	0.68	7.66	0.130	3.92	*4.41	0.65
	3.00-4.00		5.31	5.01	0.77	7.42	0.130	4.11	3.91	0.76

* Average 3.95.

† Average .74.

(Continued)

CALORIES				BODY TEMPERATURE			MORN- ING WEIGHT	BEHAVIOR OF DOG	REMARKS
metn	Non-Protein	Total Calculated	Total Found	Start	End	Difference			
1.98	12.96	16.94	*19.12	37.94	38.22	+0.28	kg. 9.3	Very quiet.	20 grams dextrose in 150 cc. water at noon.
2.98	12.91	16.89	17.24		38.16	-0.06		Quiet.	
2.98	11.77	15.75	16.56		38.04	-0.12		Quiet.	
		49.58	52.92						
5.30	13.42	18.72	18.60	38.20	38.32	+0.12	9.4	Very quiet.	20 grams dextrose in 150 cc. water at noon.
5.30	10.13	15.43	16.09		38.20	-0.12		Very quiet.	
		34.15	34.69						
4.29	10.66	14.95	14.13	38.03	38.01	-0.02	8.5	Quiet.	50 grams dextrose in 150 cc. of water at noon.
3.62	16.32	19.94	19.86	38.69	38.72	+0.03		Active 2 minutes.	
3.62	19.39	23.01	21.78		38.92	+0.20		Active 10 minutes.	
3.62	22.41	26.03	24.06		38.85	-0.07		Active 23 minutes.	
3.62	13.47	17.09	17.12		38.62	-0.23		Quiet.	
		101.02	106.95						
3.80	15.86	19.66	18.69	38.59	38.81	+0.22	8.5	Quiet.	
3.80	15.83	19.63	18.83		39.05	+0.24		Quiet.	
		39.29	37.52						
3.51	14.72	18.23	19.26	38.61	38.69	+0.08	8.5	Very quiet.	75 grams dextrose in 200 cc. of water.
3.51	13.10	16.61	17.25		38.59	-0.10		Moving 2 minutes.	
		34.84	36.51						
3.98	15.61	19.59	19.90	38.59	38.88	+0.29	8.6	Very quiet.	
3.98	15.49	19.47	18.45		38.84	-0.04		Very quiet.	
		39.06	38.35						
2.91	16.69	19.60	20.85	37.97	38.24	+0.27	9.3	Very quiet.	
2.91	17.31	20.22	21.28		38.42	+0.18		Restless 3 minutes.	
2.91	18.18	21.09	20.38		38.25	-0.17		Very quiet.	200 cc. of water.
2.91	17.33	20.24	19.44		38.33	+0.08		Quiet.	
		81.15	81.95						
3.45	13.07	16.52	17.22	38.73	38.64	-0.09	8.8	Very quiet.	
3.45	13.07	16.52	17.69		38.61	-0.03		Active 1 minute.	
3.45	13.01	16.46	17.34		38.69	+0.08		Quiet.	
		49.50	52.25						

*Heat eliminated = 17.01.

TABLE II.

DATE	TIME	EXP. NO.	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN		
								CO ₂	O ₂	R. Q.
January 23, '12	1.00-2.00 p.m.	14	grams	grams		grams	grams	grams	grams	
	2.00-3.00		5.86	4.65	0.92	19.48	0.124	4.69	3.60	0.95
			5.80	5.39	0.78	19.21	0.124	4.62	4.34	0.77
February 1, '12	2.00-3.00 p.m.	19	5.06	5.03	0.73	8.15	0.130	3.85	3.93	0.71
	3.00-4.00		5.07	5.23	0.70	7.64	0.130	3.85	4.13	0.68
January 24, '12	1.00- 2.00 p.m.	15	6.00	4.29	1.02	8.86	0.219	3.96	2.44	1.18
	2.00- 3.00		5.92	5.00	0.86	8.94	0.219	3.85	3.15	0.89
	3.00- 4.00		5.45	4.94	0.80	8.13	0.219	3.41	3.09	0.80
February 7, '12	1.00- 2.00 p.m.	23	6.05	lost		7.49	0.139			
	2.00- 3.00		5.91	lost		7.69	0.139			
February 8, '12	1.00- 2.00 p.m.	24	5.81	5.36	0.79	7.17	0.129	4.58	4.27	0.78
	2.00- 3.00		6.61	6.03	0.80	7.43	0.129	5.39	4.94	0.79

‡ See text.

(Continued)

CALORIES				BODY TEMPERATURE			MORN- ING WEIGHT	BEHAVIOR OF DOG	REMARKS
Wt.	Non-Protein	Total Calculated	Total Found	Start	End	Difference			
1.29	12.56	15.85	20.42	38.63	38.41	-0.22	kg. 8.7	Very quiet.	} 17 grams urea in 150 cc. water (vomited).
1.29	14.47	17.76	18.75		38.16	-0.25		Active 5 minutes.	
		33.61	39.17						
1.45	12.90	16.35	15.94	39.02	38.71	-0.31	8.6	Very quiet.	} 12 grams urea in 150 cc. water.
1.45	13.23	16.68	16.07		38.55	-0.16		Very quiet.	
		33.03	32.01						
5.81	9.28	15.09	18.00	38.78	38.70	-0.08	8.7	Quiet.	} 6.7 gms. NaCl in 150 cc. water.
5.81	10.83	16.64	17.37		38.64	-0.06		Active 2 minutes.	
5.81	10.38	16.19	16.54		38.62	-0.02		Quiet.	
		47.92	51.91						
			18.71	38.59	38.80	+0.21		Very quiet.	50 grams olive oil.
			18.05		38.84	+0.04		Very quiet.	
			36.76						
3.42	14.27	17.69	19.77	38.48	38.76	+0.28		Quiet.	50 grams olive oil.
3.42	16.29	19.71	19.73		39.04	+0.28		Quiet.	
		37.40	39.50						

Experiment 15—6.7 grams NaCl in 150 cc. of water.

TIME	CALORIES	
	Calculated	Found
1.00-2.00 p.m.	15.09	18.00
2.00-3.00	16.64	17.37
3.00-4.00	16.19	16.54
	47.92	51.91

These figures contradict the statement of Zuntz¹⁰ regarding the behavior of sodium chloride on metabolism. The results in no way differ from the average basal metabolism of 16.2 calories per hour. Hence, *the ingestion of 150 cc. of a solution containing 4.2 per cent sodium chloride is without influence upon the metabolism.*

The urinary excretion of sodium chloride during the experimental period between 12.00 noon and 4.08 p.m. amounted to 0.805 gram per hour and from 4.08 to 10 a.m. the next day to 0.439 gram per hour. The quantity of urine was 140 cc. during the experimental period of four hours, indicating marked diuresis.

H. The ingestion of fat.

Since dextrose solutions caused an increased metabolism and solutions such as 8 per cent urea and 4.2 per cent sodium chloride and water itself had no effect, it seemed desirable to test the influence of another food-stuff, fat, which is quickly absorbed, enters the blood current and supplies the cells with nutriment.

Fifty grams of olive oil were mixed with 10 cc. of a 1.2 per cent sodium carbonate solution and shaken till a fine emulsion formed, and this was given to the dog at noon.

The results may be thus summarized (details, Appendix, Table II).

*Experiment 23—50 grams olive oil, at noon.**Experiment 24—50 grams, olive oil, at noon.*

TIME	CALORIES		CALORIES	
	Calculated*	Found	Calculated	Found
1.00-2.00 p.m.		18.71	17.69	19.77
2.00-3.00		18.05	19.71	19.73
		36.76	37.40	39.50

*Oxygen lost, but the CO₂ per hour was 6.05 and 5.91 against 5.81 in experiment 24, on day following, during which hour the calculated metabolism was 17.69, the R. Q. being 0.79. Given these latter conditions, the calculated metabolism of experiment 23 would have been 18 calories and over in the two hours recorded.

¹⁰ Zuntz: *Zentralbl. f. Physiol.*, xxiii, p. 960, 1909.

These experiments illustrate an increased metabolism above the basal level of 16.2 calories per hour, due to the inflow of fat. If one again recalls Benedict's experiments showing the negative effect of cathartics and of agar-agar upon the metabolism, one cannot attribute this increase to "Darmarbeit." The emulsified fat flows partly through the portal vein, but in large quantity enters directly into the circulation through the thoracic duct, at once affording a means of enrichment of the fat content of the general circulation. Under these circumstances of ample nutrition, the metabolism increases (in the sense of Voit).

The general results obtained from the experiments mentioned in this paper are shown in the accompanying chart.

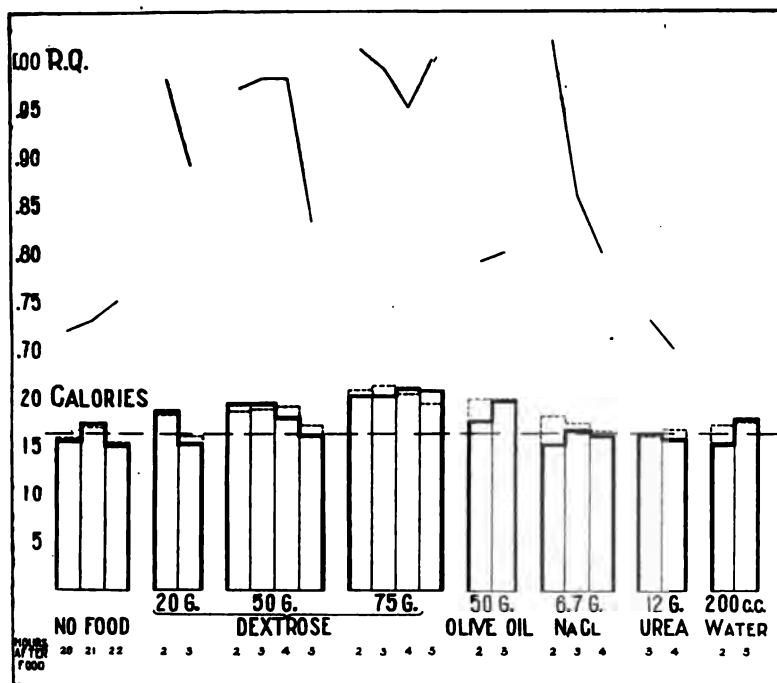


CHART 1 ILLUSTRATING THE EFFECT OF THE INGESTION OF DEXTROSE AND FAT AND OF WATER, UREA AND SALT SOLUTIONS ON THE METABOLISM.

Solid lines—metabolism in calories as calculated. Broken lines—metabolism in calories as found.

III. SUMMARY.

It is impossible to discuss the present work without anticipatory reference to the results of Miss Fisher and Miss Wishart, which are to be presented in the paper which follows this. These authors have found that after giving 50 grams of dextrose to a dog, the blood sugar rises in percentage within one hour and then falls to normal during the succeeding hours; that there is a large retention of water by the organism during the period of high metabolism, which water is suddenly eliminated during the last hour of the high metabolism (the fourth after dextrose ingestion); that during this same hour, the absorption of dextrose is completed; and that, during the period of high metabolism and water retention, the percentage of hemoglobin falls in the blood, rising again after the water elimination.

In general, the following picture may be drawn. It was noted in Dog I, that, after giving 100 grams of dextrose, the skin temperature rose during the second hour, while the rectal temperature fell slightly (second paper). When 50 grams of dextrose are given (Dog II) the sugar content of the blood in per cent is first increased, but by the end of the second hour and thereafter, it is found to have returned to its normal percentage value. The volume of the blood, however, increases during the second hour, obviously in response to the osmotic pressure exerted by the increased sugar concentration. The increase in blood volume is demonstrated by the fall in the hemoglobin content of the blood. During this period, the metabolism shows an increase of 20 per cent above the normal basal value. During the fourth hour there is final absorption of all the dextrose ingested, the metabolism remains high and there is a large excretion of water in the urine. At the end of the same fourth hour, the blood sugar content is normal, indicating that, during the withdrawal of water and the concentration of the blood, the liver and muscles maintain the normal sugar content of the blood. During the second, third and fourth hours, the high metabolism is accompanied by respiratory quotients of about 1.00 (after allowing for the influence of protein metabolism). However, during the fifth hour, when the metabolism has returned to its basal value, a non-protein respiratory quotient of 0.83 indicates that a mixture of fat and carbohydrate is being oxidized. The glycogenic function apparently enters into control and reduces the amount of carbohydrate available for combustion, and fat is oxidized as well.

It is interesting to recall in this connection that Hári¹¹ found that the respiratory quotient was increased in a fasting animal after giving adrenalin. Adrenalin causes a discharge of sugar from the glycogen repositories of the organism,¹² the percentage of blood sugar rises largely and, in virtue of this, carbohydrate tends to replace fat in the oxidative processes.

If 20 grams of dextrose be given, a rise of 15 per cent may take place during the second hour after sugar ingestion, to be followed by a normal basal metabolism in subsequent hours.

If 75 grams of dextrose be given, a 20 per cent increase in metabolism continues from the second through the fifth hour, during which hour the volume of urine becomes large, indicating that this is the final hour of the higher metabolism.

It is shown that ingestion of 200 cc. of water, of 150 cc. of a 4.2 per cent solution of sodium chloride or of 150 cc. of an 8 per cent solution of urea have no influence upon metabolism. It is, therefore, concluded that the high metabolism after giving dextrose is not due to osmotic changes between the blood and the tissues. The only remaining conclusion which appears possible is that *the increase in metabolism is due to the presence of a greater amount of free diffusible carbohydrate than is present when there is no absorption of carbohydrate from the intestines.* It is known that the sugar of the blood behaves as if it existed in chemical combination (Loewi; Lépine). If this combining power over sugar remains constant, then when the blood is diluted, with coincident maintenance of the normal percentage content of dextrose, there would be an increased supply of free readily oxidizable sugar molecules available for the nutrition of the cells. Hence, carbohydrate alone is oxidized, and the metabolism rises in virtue of an increased supply of nutriment.

An emulsion of olive oil ingested by the dog caused an increase in metabolism, probably for similar reasons.

These experiments confirm the work of Zuntz and of Rubner in many particulars, but they bring to light new points which show that the theories of both Zuntz and Rubner will have to be revised.

The views here expressed, however, conform to the "older" view of Carl Voit, who believed that the presence of abundant food increased the power of the cells to metabolize.

¹¹ Hári: *Biochem. Zeitschr.*, xxxviii, p. 23, 1912.

¹² Ringer: *Journ. of Exp. Med.*, xii, p. 105, 1910.

TABLE I.

DATE	TIME	EXP. NO.	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN		
								CO ₂	O ₂	R. Q.
May 4, '11.....	10.00-11.00 a.m.	34	grams	grams		grams	grams	grams	grams	
	11.00-12.00		10.85	7.94	0.99	9.27	0.213	8.86	6.14	1.02
	12.00-1.00 p.m.		10.68	8.15	0.95	9.06	0.213	8.65	6.35	0.99
	1.00-2.00		11.30	8.59	0.96	9.28	0.213	9.28	6.79	0.99
			11.23	8.64	0.95	9.31	0.213	9.20	6.84	0.98

TABLE II.

DATE	TIME	EXP. NO.	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN		
								CO ₂	O ₂	R. Q.
December 2, '11	9.57-10.57 a.m.	3	grams	grams		grams	grams	grams	grams	
			5.61	5.21	0.76	9.87	0.133	4.03	4.09	0.72
January 9, '12	10.00-11.00 a.m.	10	5.40	4.72	0.83	9.41	0.1331	4.14	3.50	0.86
January 22, '12	1.00-2.00 p.m.	13	5.56	4.55	0.89	8.66	0.124	4.40	3.50	0.92
	2.00-3.00		5.26	4.47	0.86	8.25	0.124	4.11	3.42	0.87
January 26, '12	1.00-2.00 p.m.	17	4.75	4.83	0.72	7.31	0.095	3.89	4.03	0.70
	2.00-3.00		5.26	5.35	0.73	7.50	0.095	4.40	4.55	0.70
	3.00-4.00		4.84	4.67	0.75	7.23	0.095	3.96	3.87	0.74
January 30, '12	1.00-2.00 p.m.	18	5.14	5.12	0.73	8.60	0.130	3.92	4.02	0.71
	2.00-3.00		5.14	4.78	0.78	7.97	0.130	3.92	3.78	0.75
	3.00-4.00		5.53	5.52	0.73	8.03	0.130	4.33	4.42	0.71
February 5, '12	1.00-2.00 p.m.	21	5.32	4.60	0.84	6.51	0.094	4.44	3.80	0.85
	2.00-3.00		5.11	4.88	0.76	6.27	0.094	4.22	4.08	0.75
	3.00-4.00		5.61	5.61	0.73	6.54	0.094	4.73	4.81	0.72
April 22, '12...	12.00-1.00 p.m.	51	5.03	4.38	0.84	10.29	0.132	3.80	3.26	0.85
	1.00-2.00		4.98	4.60	0.79	8.99	0.132	3.75	3.48	0.78
	2.00-3.00		5.23	5.12	0.74	8.18	0.132	4.00	4.00	0.73

In experiments 3 and 10 standard diet was given at noon, in the others at 6 p. m. daily.

DOG I.

CALORIES				BODY TEMPERATURE			MORNING WEIGHT	FOOD
Protein	Non-Protein	Total Calculated	Total Found	Start	End	Difference		
5.64	21.70	27.34	26.04	37.82	38.15	+0.33	Kilograms 16.3	103 grams dextrose + 400 cc. water at 9.15 a.m.
5.64	22.42	28.06	26.42		38.19	+0.04		
5.64	24.03	29.67	26.22		38.08	-0.11		
5.64	23.85	29.49	31.37		38.45	+0.37		
		-114.56	110.05					

DOG II.

CALORIES				BODY TEMPERATURE			MORNING WEIGHT	BEHAVIOR OF DOG	REMARKS
Protein	Non-Protein	Total Calculated	Total Found	Start	End	Difference			
4.07	13.46	17.53	17.61	37.89	37.85	-0.04	kg. 9.3	Very quiet.	No food.
3.51	11.94	15.45	14.54	38.44	38.44		8.9	Quiet.	No food.
3.29	12.12	15.41	14.47	38.66	38.45	-0.21	8.7	Very quiet.	No food.
3.29	11.70	14.99	17.18		38.48	+0.03		Active 7 minutes.	
		30.40	31.65					O ₂ low.	
2.52	13.21	15.73	15.89	38.36	38.26	-0.10	8.8	Very quiet.	No food.
2.52	14.92	17.44	16.40		38.27	+0.01		Slight movements.	
2.52	12.80	15.32	15.42		38.22	-0.05		Moving 1 minute.	
		48.49	47.71						
3.45	13.20	16.65	14.22	38.43	38.32	-0.11	8.8	Quiet.	No food.
3.45	12.54	15.99	14.98		38.28	-0.04		Occasional movement.	
3.45	14.51	17.96	16.99		38.43	+0.15		Active 1 minute.	
		50.60	46.19						
2.50	12.93	15.43	15.11	38.40	38.31	-0.09	9.0	Very quiet.	No food.
2.50	13.53	16.03	16.37		38.44	+0.13		Occasional movements.	
2.50	15.83	18.33	17.35		38.62	+0.18		Active 2 minutes.	
		49.79	48.83						
3.58	11.09	14.67	16.10	37.59	37.58	-0.01	9.4	Very quiet.	No food.
3.58	11.64	15.22	15.51		37.51	-0.07		Very quiet.	
3.58	13.20	16.78	17.26		37.64	+0.13		Quiet.	
		46.67	48.87						

TABLE II.

DATE	TIME	EXP. NO.	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN		
								CO ₂	O ₂	R. Q.
March 26, '12.	1.00-2.00 p.m.	39	grams 6.98	grams 4.87	1.04	grams 7.11	grams 0.150	grams 5.58	grams 3.60	1.12
	2.00-3.00		6.22	4.97	0.91	7.33	0.150	4.82	3.70	0.95
	3.00-4.00		5.49	4.72	0.84	7.04	0.150	4.09	3.45	0.86
March 30, '12.	1.00-2.00 p.m.	42	7.46	5.54	0.98	9.08	0.200	5.59	3.75	1.08
	2.00-3.00		5.74	4.68	0.89	8.63	0.200	3.87	2.89	0.97
January 3, '12.	10.20-11.20 a.m.	9	5.27	4.47	0.86	9.08	0.161	3.78	3.10	0.89
	1.00-2.00 p.m.		7.69	5.77	0.97	10.76	0.136	6.42	4.62	1.01
	2.00-3.00		8.34	6.76	0.90	10.89	0.136	7.04	5.61	0.91
	3.00-4.00		9.63	7.59	0.92	12.51	0.136	8.36	6.44	0.94
	4.00-5.00		6.27	5.02	0.91	10.30	0.136	4.99	3.87	0.94
January 12, '12	1.00-2.00 p.m.	11	7.59	5.70	0.97	8.71	0.143	6.23	4.49	1.00
	2.00-3.00		7.65	5.69	0.98	8.28	0.143	6.31	4.48	1.01
January 18, '12	3.00-4.00 p.m.	12	7.10	5.27	0.98	9.22	0.133	5.87	4.15	1.03
	4.00-5.00		5.71	4.99	0.83	8.62	0.133	4.44	3.87	0.83
February 2, '12	1.00-2.00 p.m.	20	7.41	5.71	0.94	8.41	0.150	6.01	4.44	0.98
	2.00-3.00		7.25	5.70	0.93	8.49	0.150	5.87	4.43	0.96
March 27, '12.	1.00-2.00 p.m.	40	7.83	5.66	1.01	8.19	0.115	6.76	4.69	1.05
	2.00-3.00		7.82	5.79	0.99	7.91	0.115	6.75	4.82	1.01
	3.00-4.00		8.01	6.14	0.95	8.39	0.115	6.94	5.17	0.98
	4.00-5.00		8.07	5.84	1.00	7.99	0.115	7.00	4.87	1.04
January 25, '12	1.00-2.00 p.m.	16	5.31	4.60	0.84	7.63	0.130	4.11	*3.50	0.85†
	2.00-3.00		5.13	5.51	0.68	7.66	0.130	3.92	*4.41	0.65†
	3.00-4.00		5.31	5.01	0.77	7.42	0.130	4.11	3.91	0.76

* Average 3.95.

† Average .74.

(Continued)

CALORIES				BODY TEMPERATURE			MORN- ING WEIGHT	BEHAVIOR OF DOG	REMARKS
metn	Non-Protein	Total Calcu- lated	Total Found	Start	End	Differ- ence			
3 98	12.96	16.94	*19.12	37.94	38.22	+0.28	kg. 9.3	Very quiet.	20 grams dextrose in 150 cc. water at noon.
3 98	12.91	16.89	17.24		38.16	-0.06		Quiet.	
3 98	11.77	15.75	16.56		38.04	-0.12		Quiet.	
		49.58	52.92						
5 30	13.42	18.72	18.60	38.20	38.32	+0.12	9.4	Very quiet.	20 grams dextrose in 150 cc. water at noon.
5 30	10.13	15.43	16.09		38.20	-0.12		Very quiet.	
		34.15	34.69						
4 29	10.66	14.95	14.13	38.03	38.01	-0.02	8.5	Quiet.	50 grams dex- trose in 150 cc. of water at noon.
3 62	16.32	19.94	19.86	38.69	38.72	+0.03		Active 2 minutes.	
3 62	19.39	23.01	21.78		38.92	+0.20		Active 10 minutes.	
3 62	22.41	26.03	24.06		38.85	-0.07		Active 23 minutes.	
3 62	13.47	17.09	17.12		38.62	-0.23		Quiet.	
		101.02	106.95						
3 80	15.86	19.66	18.69	38.59	38.81	+0.22	8.5	Quiet.	50 grams dex- trose in 150 cc. of water at noon.
3 80	15.83	19.63	18.83		39.05	+0.24		Quiet.	
		39.29	37.52						
3 51	14.72	18.23	19.26	38.61	38.69	+0.08	8.5	Very quiet.	75 grams dex- trose in 200 cc. of water.
3 51	13.10	16.61	17.25		38.59	-0.10		Moving 2 minutes.	
		34.84	36.51						
3 98	15.61	19.59	19.90	38.59	38.88	+0.29	8.6	Very quiet.	75 grams dex- trose in 200 cc. of water.
3 98	15.49	19.47	18.45		38.84	-0.04		Very quiet.	
		39.06	38.35						
2 91	16.69	19.60	20.85	37.97	38.24	+0.27	9.3	Very quiet.	75 grams dex- trose in 200 cc. of water.
2 91	17.31	20.22	21.28		38.42	+0.18		Restless 3 min- utes.	
2 91	18.18	21.09	20.38		38.25	-0.17		Very quiet.	200 cc. of water.
2 91	17.33	20.24	19.44		38.33	+0.08		Quiet.	
		81.15	81.95						
3 45	13.07	16.52	17.22	38.73	38.64	-0.09	8.8	Very quiet.	200 cc. of water.
3 45	13.07	16.52	17.69		38.61	-0.03		Active 1 minute.	
3 45	13.01	16.46	17.34		38.69	+0.08		Quiet.	
		49.50	52.25						

*Heat eliminated = 17.01.

TABLE II.

DATE	TIME	EXP. NO.	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN		
								CO ₂	O ₂	R. Q.
January 23, '12	1.00-2.00 p.m.	14	grams	grams		grams	grams	grams	grams	
	2.00-3.00		5.86	4.65	0.92	19.48	0.124	4.69	3.60	0.95
			5.80	5.39	0.78	19.21	0.124	4.62	4.34	0.77
February 1, '12	2.00-3.00 p.m.	19	5.06	5.03	0.73	8.15	0.130	3.85	3.93	0.71
	3.00-4.00		5.07	5.23	0.70	7.64	0.130	3.85	4.13	0.68
January 24, '12	1.00- 2.00 p.m.	15	6.00	4.29	1.02	8.86	0.219	3.96	2.44	1.18
	2.00- 3.00		5.92	5.00	0.86	8.94	0.219	3.85	3.15	0.89
	3.00- 4.00		5.45	4.94	0.80	8.13	0.219	3.41	3.09	0.80
February 7, '12	1.00- 2.00 p.m.	23	6.05	lost		7.49	0.139			
	2.00- 3.00		5.91	lost		7.69	0.139			
February 8, '12	1.00- 2.00 p.m.	24	5.81	5.36	0.79	7.17	0.129	4.58	4.27	0.78
	2.00- 3.00		6.61	6.03	0.80	7.43	0.129	5.39	4.94	0.79

‡ See text.

(Continued)

CALORIES				BODY TEMPERATURE			MORN- ING WEIGHT	BEHAVIOR OF DOG	REMARKS
Protein	Non-Protein	Total Calculated	Total Found	Start	End	Difference			
3.29	12.56	15.85	20.42	38.63	38.41	-0.22	8.7	Very quiet.	} 17 grams urea in 150 cc. water (vomited).
3.29	14.47	17.76	18.75		38.16	-0.25		Active 5 minutes.	
		33.61	39.17						
3.45	12.90	16.35	15.94	39.02	38.71	-0.31	8.6	Very quiet.	} 12 grams urea in 150 cc. water.
3.45	13.23	16.68	16.07		38.55	-0.16		Very quiet.	
		33.03	32.01						
5.81	9.28	15.09	18.00	38.78	38.70	-0.08	8.7	Quiet.	} 6.7 gms. NaCl in 150 cc. water.
5.81	10.83	16.64	17.37		38.64	-0.06		Active 2 minutes.	
5.81	10.38	16.19	16.54		38.62	-0.02		Quiet.	
		47.92	51.91						
			18.71	38.59	38.80	+0.21		Very quiet.	50 grams olive oil.
			18.05		38.84	+0.04		Very quiet.	
			36.76						
3.42	14.27	17.69	19.77	38.48	38.76	+0.28		Quiet.	50 grams olive oil.
3.42	16.29	19.71	19.73		39.04	+0.28		Quiet.	
		37.40	39.50						

ANIMAL CALORIMETRY.

FOURTH PAPER.

OBSERVATIONS ON THE ABSORPTION OF DEXTROSE AND THE EFFECT IT HAS UPON THE COMPOSITION OF THE BLOOD.

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In the experiments described in the last paper, two results were noted. In the first place, the temperature of the skin rose to a greater degree after giving dextrose than did the rectal temperature, and in the second place, the period of high metabolism was terminated by an hour in which a large quantity of water was eliminated in the urine. The first observation pointed to a change in the distribution of the blood, whereas the second indicated that the volume of the blood had been increased during the period of the higher metabolism, only to diminish again through the sudden excretion of water at the end of the period. This paper presents results designed to follow these facts to a definite interpretation.

An old observation of Bischoff and Voit¹ noted that a dog which had been fed with bread for forty-one days, during which time the urinary nitrogen indicated a loss of 3.7 kgms. of "flesh," in reality lost only 0.5 kgm. in weight, and when 1800 grams of meat were given to the animal, water was eliminated in larger quantity in the urine than was taken in the food. Although the fact of an increased quantity of water held by the tissues when an animal is maintained on a carbohydrate diet, has been repeatedly confirmed, there has been no analysis of this condition extending over a short period of time.

¹ Bischoff and Voit: *Die Gesetze der Ernährung des Fleischfressers*, 1860, p. 211.

50 Dextrose Absorption and Blood Composition

Experiments which describe the effect of dextrose ingestion upon the content of blood sugar have been accomplished on man. Recent determinations by E. Frank² give values of 0.07 to 0.09 per cent of dextrose in normal human blood. Liefmann and Stern³ state that 0.105 per cent represents the maximum amount of dextrose normally present. However, these authors note that in two patients with croupous pneumonia, the dextrose content of the blood was 0.108 and 0.136 per cent, which, however, rose one hour after the ingestion of 200 grams of dextrose to 0.17 and 0.281 per cent respectively. Gilbert and Baudouin⁴ have given 150 grams of dextrose in 400 cc. of water to six normal young men. They report that the blood sugar rose after one hour and amounted to 0.107, 0.108, 0.125, 0.130, 0.132 and 0.134 per cent in the six individuals. After two hours the blood sugar content was only slightly above the normal, and in the third and fourth hours it was once more entirely normal.

In addition to these experiments, the procedure in which is entirely comparable to that employed by the present writers, other experiments have been performed in which dextrose solutions were injected directly into the venous system and the results noted. Thus Brasol,⁵ in the laboratory of Carl Ludwig, found that two minutes after intravenous injection of dextrose solutions, much of the injected dextrose was already removed from the blood, the percentage of hemoglobin fell, indicating a dilution due to osmotic processes, but in two hours the normal percentage of sugar had been reached. Repeating these experiments, Nadporozsky⁶ found that, following intravenous injection of dextrose solutions, the hemoglobin at first fell but after forty-five minutes exhibited normal relations. Starling⁷ injected 40 grams of dextrose in 40 cc. of water into the jugular vein of a dog. Within five minutes the hemoglobin in the blood fell from 100 to 60 per cent but after half an hour returned to normal. Accompanying the hydremic

² E. Frank: *Zeitschr. f. physiol. Chem.*, lxx, p. 139, 1911.

³ Liefmann and Stern: *Biochem. Zeitschr.*, i, p. 301, 1906.

⁴ Gilbert and Baudouin: *Compt. rend. soc. biol.*, lxxv, p. 710, 1908.

⁵ Brasol: *Arch. f. Physiol.*, 1884, p. 211.

⁶ Nadporozsky: *Ruskaja Medicina*, 1887, No. 26: Abstract in *Maly's Jahresbericht für Tierchemie*, 1887.

⁷ Starling: *Journ. of Physiol.*, xxiv, p. 317, 1899; see also *The Fluids of the Body*, 1909.

plethora, due to the withdrawal of water from the tissues in consequence of osmotic differences, there was a rise in blood pressure, an increase in kidney volume and an increase in the elimination of urine. Starling⁸ believes that the consequences of the hydremic plethora are increased circulation through the kidney and increased output of urine, the dilution of the blood favoring glomerular filtration. The urine contained dextrose and Starling also suggests that dextrose itself leads to a stimulus of the kidney vessels or possibly the kidney cells, because the diuresis outlasts the hydremic plethora. During this latter period, the quantity of hemoglobin rises above the normal on account of the continued diuresis.

Biedl and Kraus⁹ have administered intravenously, 20 and 30 grams of dextrose in 10 per cent solutions to human beings, have noted a rapid disappearance of excess of sugar in the blood, and have found that no considerable amount of sugar was eliminated in the urine. These experiments all point to the efficiency of the glycogenic function of the body which tends to maintain the dextrose content of the blood a fixed level.

EXPERIMENTAL PART.

In paper three was described how a dog (Dog II), weighing about 9 kgms., was maintained on a standard diet and how eighteen hours after food ingestion, dextrose was administered (usually in 150 cc. of water) and the metabolism then determined in hourly periods. The following experiments were designed to nearly reproduce these conditions with a view to ascertaining certain details during the different hours of dextrose absorption. The facts investigated were (a) the amount of dextrose in the gastrointestinal tract, (b) the dextrose content of the blood, (c) the glycogen content of the liver, (d) the quantity of urine secreted and (e) the hemoglobin content of the blood. The first three factors were determined upon ten different dogs (shown in Table I) while the last two factors were observed upon the same dog (shown in Table II).

Reference to Table I reveals a series of experiments upon ten dogs which were given a standard diet one or more days. In all

⁸ Starling: *The Fluids of the Body*, 1909, p. 153.

⁹ Biedl and Kraus: *Wiener klin. Wochenschr.*, ix. p. 55, 1896.

the cases blood was withdrawn under cocaine anesthesia, a method which was employed because most anesthetics lead to an output of dextrose in the urine. The cocaine was first used in a 7 per cent solution, which proved too strong and led to the death of the animal in convulsions. With a 1 per cent solution of cocaine there was no difficulty throughout the entire series of experiments, except in the case of a small nervous dog (IV). In the other instances, after the subcutaneous administration of cocaine immediately over the carotid artery, a canula could be inserted in the vessel and two samples of blood of about 50 cc. each could be drawn from the perfectly quiet animal without any sign of pain or irritation. The dog was then killed by a sudden blow on the head, the liver quickly removed and the stomach and intestinal contents were covered with 95 per cent alcohol.

The blood sugar was determined by the method of Weymouth Reid¹⁰ as used by Vosburgh and Richards.¹¹ Glycogen in the liver was determined according to Pflüger¹² and all sugar determinations according to the method of Allihn. The Fleischl-Miescher method was employed in the hemoglobin tests.

a. The quantity of dextrose in the gastro-intestinal tract.

In five cases, when the animal was killed twenty-four hours after administration of the standard diet, the gastro-intestinal tract was found to be free from dextrose. When 50 grams of dextrose were given about twenty-four hours after the standard diet, a rapid though variable absorption of dextrose was recorded. The results may thus be summarized.

Rate of absorption after giving 50 grams of dextrose.

TIME AFTER FOOD	DEXTROSE	
	In stomach	In intestine
	grams	grams
One hour.....	5.6	2.9
Two hours.....	11.1	1.7
Two hours.....	16.6	1.0
Three hours.....	7.4	0.0
Four hours.....	0.0	0.0

¹⁰ Reid: *Journ. of Physiol.*, xx, p. 316, 1896.

¹¹ Vosburgh and Richards: *Amer. Journ. of Physiol.*, ix, p. 35, 1903.

¹² Pflüger: *Das Glycogen*, 2te Aufl., 1905, p. 67.

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These figures indicate that the absorption of 50 grams of dextrose in dogs weighing between 8 and 9 kgms. is rapid and is completed during the fourth hour after the administration of the sugar.

b. The dextrose content of the blood.

It has been repeatedly verified that convulsions increase the quantity of blood sugar through depletion of the glycogen reserves of the body. This is apparent in values of 0.19 and 0.14 per cent of dextrose contained in the blood taken during cocaine convulsions. In two cases, however, when the blood was obtained from a quiet dog twenty-four hours after food ingestion, duplicate analyses showed in one dog 0.10 and in another 0.11 per cent of glucose, which are entirely normal values.

After giving 50 grams of dextrose in 150 cc. of water to dogs which had received the standard diet twenty-four hours before, the following results were obtained.

Time after 50 grams dextrose.	Blood sugar in per cent.
One hour.....	0.16
*One hour.....	0.13
Two hours.....	0.10
Two hours.....	0.11
Three hours.....	0.11
Four hours.....	0.11

*Not included in Table I.

These results confirm those of Gilbert and Baudouin on man, in showing that the blood sugar rises during the first hour and then falls, remaining normal thereafter.

c. The glycogen content of the liver.

The glycogen content of the liver is known to be very variable in different dogs twenty-four hours after food ingestion, so that glycogen determinations often show little that is significant. In this series, in the two "normal" cases, there were 7.47 grams (2.97 per cent) and 7.70 grams (2.7 per cent) of glycogen in the liver. Much less was found in the dogs which were killed during convulsions, being 3.26, 5.81 and 2.28 grams (2.00, 1.48 and 1.31 per cent).

The following table shows the quantity of glycogen found in the liver at different hours after giving 50 grams of dextrose, and

this is compared with the quantity of dextrose absorbed from the intestine as actually determined.

The influence of ingesting 50 grams of dextrose on the glycogen content of the liver.

TIME AFTER 50 GRAMS OF DEXTROSE	DEXTROSE INGESTED		LIVER GLYCOGEN	
	Absorbed	Present in body but unoxidized (calculated)		
	grams	grams	grams	per cent
One hour.....	41.5	37.1	12.75	3.33
Two hours.....	37.2	28.4	7.95	3.30
Two hours.....	32.4	23.6	11.90	3.85
Three hours.....	42.6	29.0	6.21	2.56
Four hours.....	50.0	32.4	21.28	7.24

The respiration experiments in the last paper showed that about 16 calories of carbohydrate were oxidized per hour during the second and third and fourth hours in a dog similar in size to those here employed. This is the equivalent of 4.4 grams of dextrose per hour. This must be considered a minimal value as it was obtained in a dog in complete repose. Adopting this value for all the different periods described above, it is possible to estimate the maximal quantity of the absorbed dextrose which could have been present and unoxidized in the organism at the end of any given experiment.

One point stands out clearly and that is that during the period of high metabolism dextrose is not retained in the liver in any considerable quantity. The larger part of the absorbed dextrose passes to other tissues. Only at the end of four hours and after complete absorption of all the dextrose from the organism, does glycogen appear in large quantity in the liver. This fourth hour is the last hour of the high metabolism. While it is realized that it is dangerous to draw too sweeping conclusions from a single experiment, still it seems very probable that the end of the fourth hour is marked by a reduction of the flood tide of carbohydrate material to the tissues, the excess being deposited in the liver.

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d. The quantity of urine secreted.

In paper three of this series, it was noticed that after giving 50 grams of dextrose in solution to a dog, there was a large secretion of urine during the fourth hour, which terminated the period of high metabolism. In the present series of experiments, a dog was used weighing between 7 and 8 kgms. The animal was first starved for two days, then on a third day of fasting received 150 cc. of water and on the evening of that day and thereafter received the standard diet. On various occasions at noon, he received 20 grams and 50 grams of dextrose dissolved in 150 cc. of water and 75 grams of dextrose in 200 cc. of water. These experiments were designed to reproduce the conditions described in paper three.

The urine was obtained by means of a sterile catheter. At noon the bladder was washed, at first with warm water and then with a solution of boracic acid. Then, during the experimental period, the bladder was emptied at the end of each hour, without washing it. When the last portion of urine was removed, the bladder was washed with boracic acid solution to prevent infection. The results are summarized in the following table and are graphically presented in the accompanying chart.

Influence of dextrose solutions upon the hourly secretion of urine.

TIME	FAST- ING 2 DAYS	FAST- ING 3 DAYS 150 CC. WATER	STANDARD DIET 18 HOURS BEFORE FOOD				
			150 cc. water and				200 cc. water and 75 grams dextrose
			20 grams dextrose	50 grams dextrose	50 grams dextrose	50 grams dextrose	
<i>p. m.</i>							
12.00-1.00	7.0	28.0	11.5	4.5	7.0	4.0	6.5
1.00-2.00	2.0	27.0	23.0	6.0	7.5	4.0	6.0
2.00-3.00	4.5	28.0	13.0	8.5	12.0	12.5	7.0
3.00-4.00	2.5	17.5		22.0	100.0	88.5	18.5
4.00-5.00			28.5		66.5	19.0	89.0
5.00-6.00					22.0	10.0	
6.00-7.00					16.0		
Total.....	18.0	100.5		41.0	231.0	138.0	127.0

It is apparent from these figures that, if water be given alone, it is quickly eliminated. If the same quantity of water be given

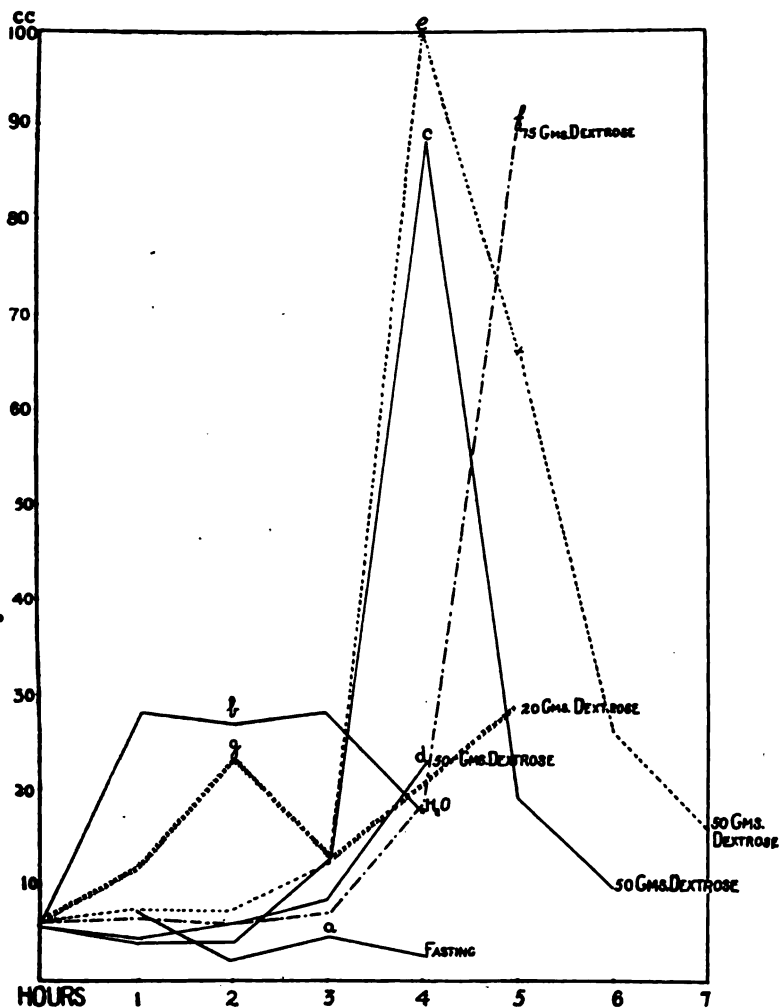


CHART ILLUSTRATING THE INFLUENCE OF INGESTED DEXTROSE SOLUTIONS UPON THE HOURLY VOLUME OF THE URINE.

Straight lines = hourly urinary secretion during (a) fasting; (b) after 150 cc. water; (c) 50 grams dextrose in 150 cc. water; (d) 50 grams dextrose in 150 cc. Dotted line (e) = 50 grams dextrose in 150 cc. water. Dash and dot line (f) = 75 grams dextrose in 200 cc. Star line (g) = 20 grams dextrose in 150 cc.

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with 20 grams of dextrose, there is at first some hindrance to its elimination. If 50 grams of dextrose be added to 150 cc. of water, the result of its ingestion is to reduce the quantity of urine eliminated during the first two hours to practically the fasting minimum; during the third hour there is a slight increase and during the fourth hour the elimination of urine rises to large volume, declines in the fifth hour and becomes greatly reduced in the sixth and seventh hours. A similar picture is presented when 75 grams of dextrose are given in 200 cc. of water, only the minimal excretion lasts three hours instead of two, there is a slight increase during the fourth hour and the large increase in volume takes place in the fifth hour. In Dog II in the previous paper, after giving 50 grams of dextrose, the fourth hour marked the end of the high metabolism and it is here found to be the hour of large urinary excretion. In the same dog, after giving 75 grams of dextrose, the high metabolism continued during the fifth hour, which corresponds to the hour of the largely increased output of urine in the present experiment.

The *nitrogen elimination* is shown in Table II. These results, however, are not as reliable as those given for Dog II in the last paper because the bladder was not washed at the end of each period, and strictly comparable results could not, therefore, be achieved. In general, however, a confirmation of the former results has been obtained; there is a small output of nitrogen during the period of diminished urine secretion and a larger output during the period of diuresis.

e. The hemoglobin content of the blood.

Making use of the same dog, blood for hemoglobin tests was obtained every hour immediately after catheterization. The blood was obtained by cutting a small ear vessel, usually an artery, and taking a sample of the freely flowing blood. The incision was reopened every hour with little difficulty and the ear was washed with an antiseptic solution after taking the blood. The determinations were made by the Fleischl-Miescher method, using accurate pipette dilutions. A count of the red blood cells was made as a check with regard to the reliability of the hemoglobin determinations. It was repeatedly found that twenty-four

TABLE II.

The effect of ingested dextrose solutions upon the quantity of urine eliminated and upon the dilution of the blood as indicated by the per cent of hemoglobin in it.

DIET	TIME	URINE PER HOUR	NITROGEN PER HOUR	HEMOGLOBIN AT END OF HOUR
	p. m.	cc.	gram	per cent
Starved 48 hours	12.00-1.00	7.0		104
	1.00-2.00	2.0		100
	2.00-3.00	4.5		99
	3.00-4.00	2.5		102
150 cc. H ₂ O at 12.00	12.00-1.00	28.0		103
	1.00-2.00	27.0		101
	2.00-3.00	28.0		101
	3.00-4.00	17.5		103
20 grams dextrose, 150 cc. H ₂ O at 12.00	12.00-1.00	11.5		99
	1.00-2.00	23.0		100
	2.00-3.00	13.0		95
	3.00-4.00	Lost		95
50 grams dextrose, 150 cc. H ₂ O at 12.00	4.00-5.00	28.5		96
	12.00-1.00	8.0		92
	1.00-2.00	6.5		83
	2.00-3.00	4.5		81
50 grams dextrose, 150 cc. H ₂ O at 12.00	3.00-4.00	9.0		97
	4.00-5.00	Lost		
	12.00-1.00	4.5	0.0730	102
	1.00-2.00	6.0	0.1039	95
50 grams dextrose, 150 cc. H ₂ O at 12.00	2.00-3.00	8.5	0.2162(?)	
	3.00-4.00	22.0	0.0969	90
	4.00-5.00			100
	12.00-1.00	7.0	0.1418	108
50 grams dextrose, 150 cc. H ₂ O at 12.00	1.00-2.00	7.5	0.1769	99
	2.00-3.00	12.0	0.1601	98
	3.00-4.00	100.0	0.1797	98
	4.00-5.00	66.5	0.1516	102
50 grams dextrose, 150 cc. H ₂ O at 12.00	5.00-6.00	22.0	0.1336	107
	6.00-7.00	16.0	0.1095	106
	12.00-1.00	4.0	0.0679	109
	1.00-2.00	4.0	0.0927	80
50 grams dextrose, 150 cc. H ₂ O at 12.00 noon	2.00-3.00	12.5	0.1683	85
	3.00-4.00	88.5	0.1572	90
	4.00-5.00	19.0	0.1292	105
	5.00-6.00	10.0	0.1404	110
75 grams dextrose, 200 cc. H ₂ O at 12.00	-12.00			102
	12.00-1.00	6.5	0.0927	100
	1.00-2.00	6.0	0.0997	100
	2.00-3.00	7.0	0.1404	101
75 grams dextrose, 200 cc. H ₂ O at 12.00	3.00-4.00	18.5	0.1263	95
	4.00-5.00	89.0	0.1544	90

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hours after food, the dog showed 100 per cent of hemoglobin in the blood or slightly over 100. The method was sufficiently reliable to demonstrate that in fasting and after giving 150 cc. of water, the hemoglobin remained constant in quantity; that, after giving 20 grams of glucose in 150 cc. of water, there was a slight fall in the amount of hemoglobin; and that giving 50 grams of dextrose in 150 cc. caused little or no effect during the first hour but during the second hour it may frequently bring about a fall of 10 to 20 per cent in the percentage of hemoglobin present in the blood. A rise to its former value takes place only after the elimination of water from the blood by the kidney. These experiments show that after giving dextrose in solution *per os*, a hydremic plethora may be established in the presence of a greatly diminished secretion of urine, being a direct contradiction of the theories of Starling already discussed, according to which the excretion of urine is greatly favored by a condition of hydremic plethora.

DISCUSSION AND SUMMARY.

After the ingestion of 50 grams of dextrose in 150 cc. of water, there is a rapid absorption of dextrose during the first hour, the sugar in the blood rises above its normal content and the hemoglobin content is not profoundly changed (the absorption of water is probably slight owing to the osmotic power of the dextrose solution in the stomach). At the end of the second hour, between two-thirds and three-quarters of the sugar ingested has been absorbed, relatively little has been retained by the liver as glycogen, the sugar percentage has become normal and the blood usually more dilute as shown by a fall in the percentage amount of hemoglobin. The dilution has taken place as a result of the increased osmotic power of the blood due to the increase in sugar content found at the end of the first hour and the metabolism is 20 per cent higher than before and is at the expense of dextrose, this being due to the generous distribution of freely dissolved and uncombined dextrose molecules to the tissues (see last paper). This condition lasts through the third hour. During the fourth hour the absorption of dextrose is completed, the urinary secretion, the volume of which has been almost at the fasting level, suddenly increases very largely, the hydremic plethora of the blood tends to diminish and this

hour is the last hour of the increased metabolism. In one instance, at the end of the fourth hour, a large quantity of glycogen was found in the liver (a condition not found at the end of the previous hours) which suggests that the liver may have been active in the removal of the increased quantity of freely diffusible dextrose previously within the blood tissues. With the return of the blood to its normal volume, the percentage content of dextrose is not altered through the concentration of the blood. The fifth hour, marked by the return of the metabolism to its basal value, and which may be characterized by a lowered respiratory quotient, shows a lessened quantity of urinary output and a normal or above normal hemoglobin percentage in the blood.

With 20 grams of sugar these reactions are much less marked.

With 75 grams of sugar the high metabolism and the diminution of the volume of urinary secretion lasts an hour longer than with 50 grams of dextrose.

STUDIES IN BACTERIAL METABOLISM. VII.

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(Received for publication, July 17, 1912.)

A most fundamental principle of bacterial metabolism may be expressed concisely by stating that "Fermentation takes precedence over putrefaction;"¹ that is to say, bacteria in general which can utilize both carbohydrate and protein, act upon the former in preference to the latter when both are present in the same medium.

In view of the confusion attending the use of the terms putrefaction and fermentation, they must be sharply defined. By fermentation is meant "the action of microorganisms upon carbohydrate," and by putrefaction is meant "the action of microorganisms upon nitrogenous substances."²

Bacteria in common with all known living things need nitrogen to build up their bodies; it is self-evident, therefore, that even when carbohydrate is being fermented, enough protein must be broken down to satisfy their nitrogen requirements. Bacterial activity, therefore, must be sharply differentiated into two distinct processes, the structural and the vegetative, both functions being essential for their metabolism.

Nitrogen is indispensable for the structural process, hence bacteria must have nitrogen in their dietary. With the vast majority of bacteria, however, the vegetative process may be satisfied either by utilizable carbohydrate or by protein. Whenever bacteria can utilize both carbohydrate and protein for their vegetative activity (for fuel) and both are present in the medium in which these

¹ Kendall: *Journ. Amer. Med. Assoc.*, lvi, pp. 1084-1088.

² For a more complete discussion of these terms, see Kendall: *Journ. of Med. Res.*, pp. 140-144, 1911.

organisms are growing, the carbohydrate is invariably selected in preference to the protein. Even when protein is being utilized for vegetative purposes, the bacteria actually eliminate nitrogen from the protein molecule and apparently utilize only the carbon, hydrogen and oxygen for their energy metabolism (fuel). This amounts practically to the use of carbohydrate in the last analysis for fuel purposes.

In the past, but little effort has been made to study bacterial metabolism quantitatively, at least from the comparative standpoint, yet it is largely from the comparative study of different types of bacterial metabolism that the fundamental principles can be elucidated. This lack of diligence cannot be explained wholly by the inadequateness of the older methods; it is rather attributable to rigid adherence to the narrow, botanical idea of morphology and differentiation of bacteria instead of the broader, dynamical consideration of bacterial activity. The few painstaking studies which have been made along these lines have failed for the most part because of the neglect of the carbohydrate factor in the media in which such experiments have been carried out. For these reasons, it is impossible to quote from the literature any studies which are carried out along lines similar to those presented below.

The results upon which this summary is based have been derived from the comparative study of a considerable number of bacterial types using methods of far greater accuracy than those previously available. The types of bacteria represented in this series cover those commonly met with in bacteriology, and a sufficiently large number of strains of each type have been examined to give definite assurance of the physiological and chemical limits of each species. The organisms selected for this work have been drawn largely from the normal and pathological flora of the gastro-intestinal tract where dietary alternations, comparable with those to which these bacteria are subjected culturally, are commonly met with.

The methods employed have been described critically in previous communications,³ and will not be referred to here other than to state that the limits of error are much less than the thickness of the lines shown in the various curves at the end of the paper.

Similarly, the analytical results have been presented in previous

³ Folin and Farmer: this *Journal*, xi, p. 493; Folin and Macallum: *ibid.*, xi, p. 523; Kendall and Farmer: *ibid.*, xii, pp. 13, 19, 1912.

articles.⁴ It is the purpose of this communication to plot, analyze and synthesize these results. The appended curves, accurately constructed from these analytical figures, show graphically the effects of carbohydrate upon bacterial metabolism. These curves furthermore illustrate in a striking manner the diversity of types of bacterial metabolism in media of the same composition; yet in spite of these diversities the sparing action of carbohydrate for protein is apparent, except in the strictly "carnivorous" organisms, *B. alcaligenes* and H-61.

The frankly pathogenic organisms associated with toxemia in the human body, as typhoid and dysentery (both Shiga and Flexner), break down but little protein, as is shown by the small amount of ammonia liberated in the sugar-free medium, when they are using protein for fuel as well as for structural purposes. The amount of ammonia liberated by the less frankly pathogenic organisms increases progressively as the more saprophytic types, *e.g.*, *B. proteus*, are approached. At first sight, cholera might seem to be an exception to this generalization. It must be remembered, however, that cholera may be a rapidly fatal disease, the entire course from infection to death taking place within twelve hours. This contrasts strikingly with diseases such as typhoid, where the incubation period alone is about fourteen days on the average. The proteolytic activity of cholera may be effectively checked by the presence of dextrose.

It will be seen from the curves that the proteolytic activity, but not the structural activity of bacteria (except the strictly carnivorous types), can be arrested by the presence of utilizable carbohydrate. The products of proteolytic activity, which are only formed when bacteria are utilizing protein for fuel, are alkaline, nitrogenous substances; the products of fermentation, on the contrary, which are formed when bacteria are utilizing carbohydrate for fuel, are non-nitrogenous, acid products. It must be remembered that all known true toxins are nitrogenous, while acids produced by fermentation are at best but irritants and are for the most part non-nitrogenous. It would appear, therefore, that the production of toxic substances of bacterial origin must be the result of proteolytic (putrefactive) activity rather than of fermentative activity.

⁴ Kendall and Farmer: this *Journal*, xii, pp. 13, 19, 215, 219, 465, 469, 1912.

The importance of the sparing action of carbohydrate for protein in the light of toxin production must be apparent.

We believe that the principle elucidated above is not limited to bacteria alone, but that it is in reality a general and fundamental principle of cellular metabolism.

EXPLANATION OF CHARTS.

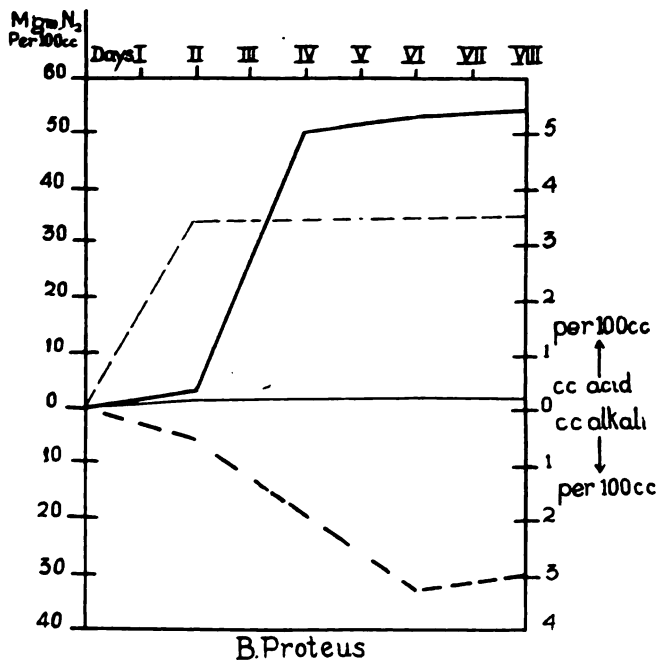
The solid, heavy line represents the production of ammonia in milligrams of nitrogen per 100 cc. of culture medium in sugar-free broth.

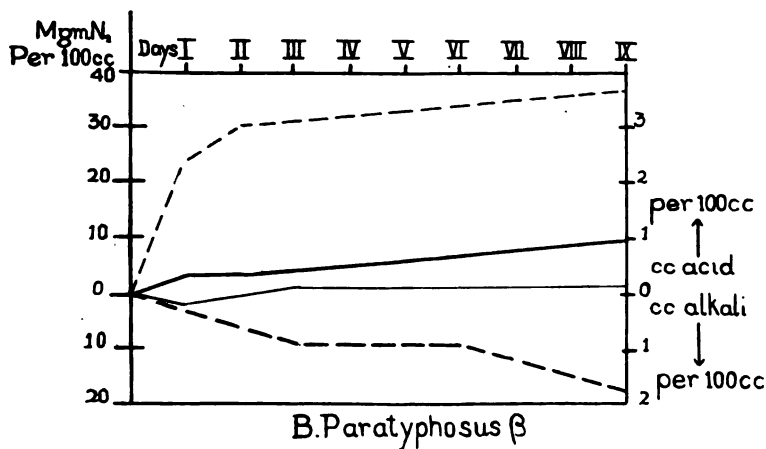
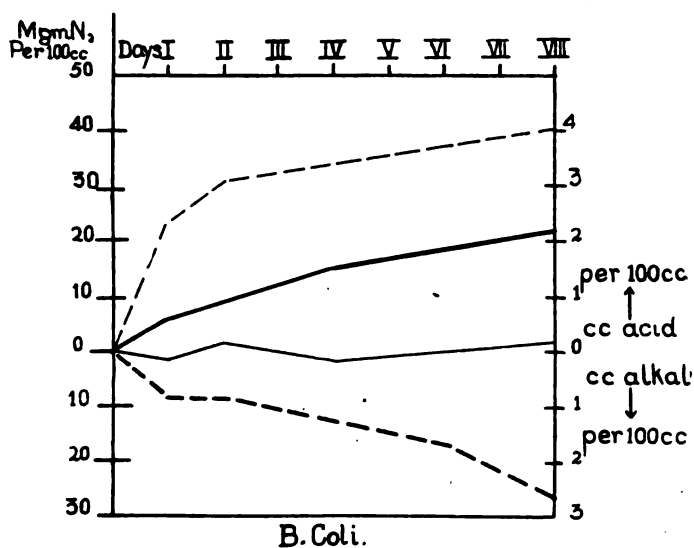
The solid, light line represents the production of ammonia in milligrams of nitrogen per 100 cc. of culture medium in dextrose broth.

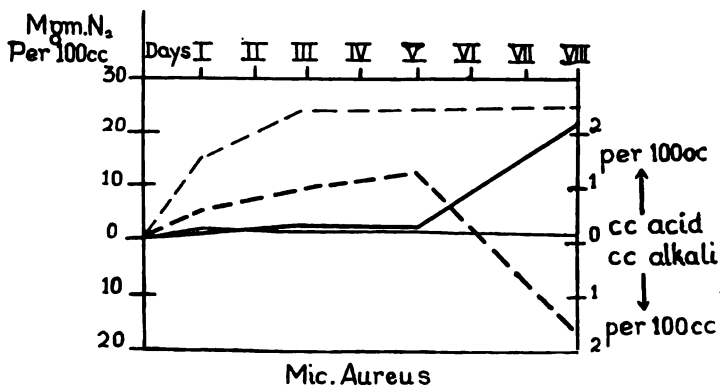
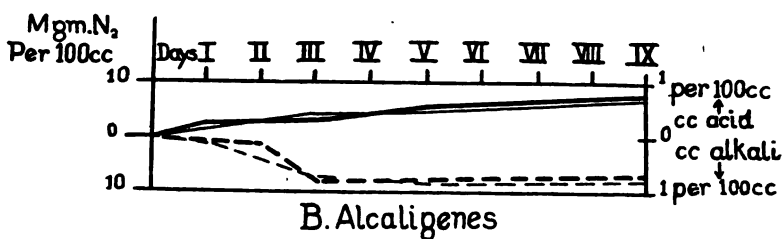
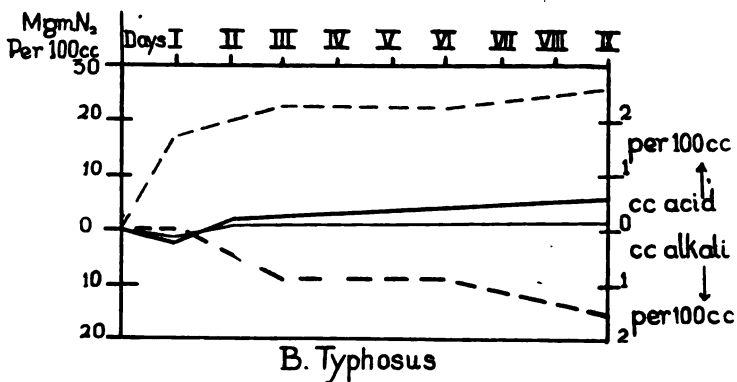
The broken heavy line represents reaction in terms of cubic centimeters of normal acid or alkali per 100 cc. of sugar-free culture medium.

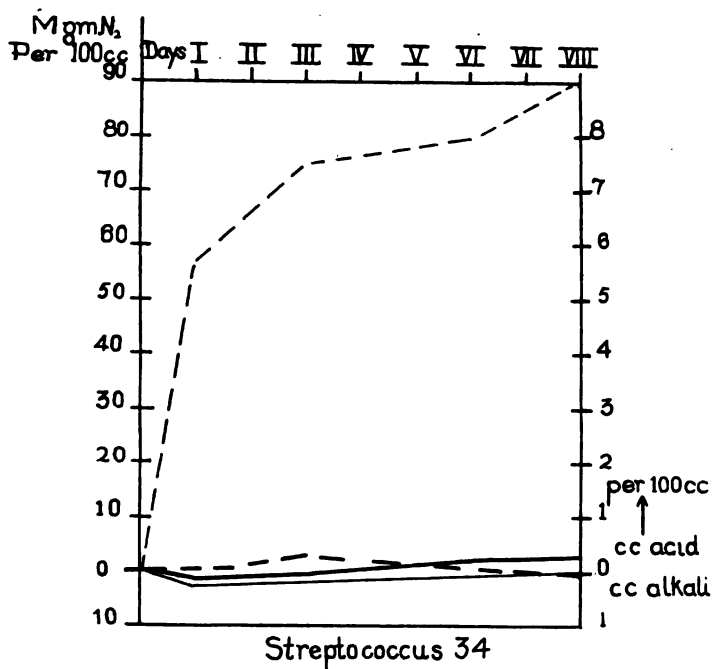
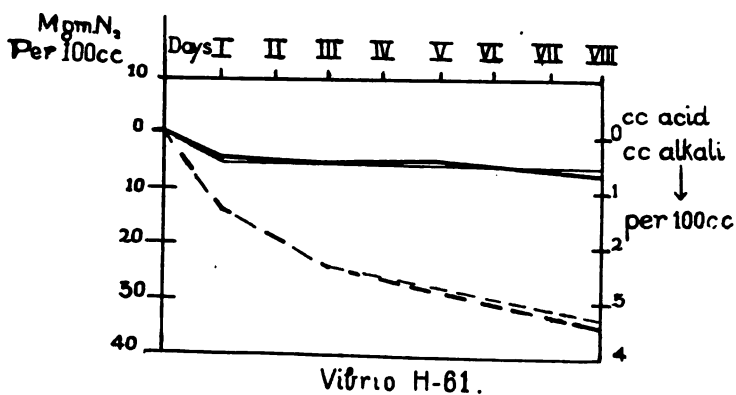
The broken light line represents reaction in terms of cubic centimeters of normal acid or alkali per 100 cc. of dextrose broth.

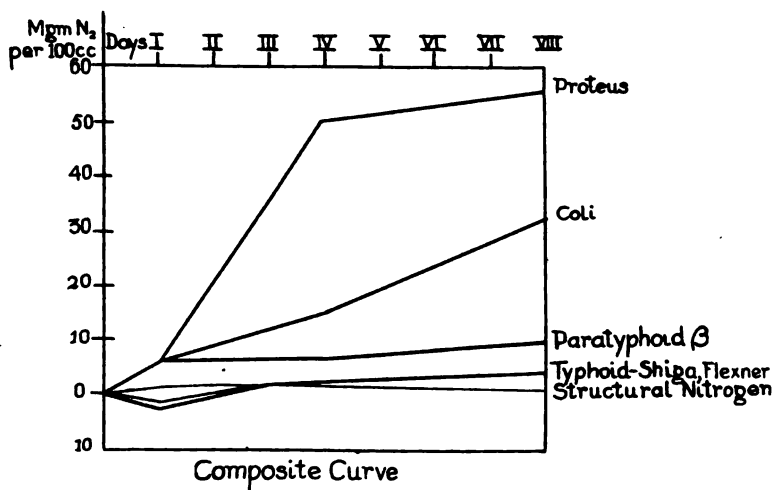
The composite curve illustrates graphically the relative amounts of ammonia produced by various types of pathogenic and saprophytic bacteria; for convenience the different types are drawn in the same figure to bring out forcibly the difference in ammonia production in sugar-free broth between these types. It will be seen that the production of ammonia in dextrose broth is essentially the same for all these organisms, excepting those which can utilize no sugar. This ammonia production in sugar-containing broth is a measure of the nitrogen needs of bacteria for structural purposes as contrasted with the fuel needs.











THE BEHAVIOR OF FAT-SOLUBLE DYES AND STAINED FAT IN THE ANIMAL ORGANISM.¹

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(Received for publication, August 26, 1912.)

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INTRODUCTION.

Since Daddi² discovered that Sudan III, when fed incorporated with fat, is absorbed and laid down in the adipose tissue of animals, various experimenters have used the dye as a means of studying problems connected with fat metabolism. The possibilities of this method have not been exhausted, and the present investigation was aimed to extend the application of fat-soluble dyes to the solution of some of the unanswered questions.

The dyes used were: Sudan III (Kahlbaum); Biebrich Scarlet (Aniline Red, R. Medicinal. Merck); Indophenol (H. A. Metz and Company); Oil Soluble Green (H. A. Metz and Company); Oil

¹ A preliminary report of some of the data recorded here was presented to the Society for Experimental Medicine and Biology (cf. *Proceedings*, viii, p. 126, 1911). The essential facts in this paper are taken from the dissertation presented by Amy L. Daniels for the degree of Doctor of Philosophy, Yale University, 1912.

² *Arch. ital. de biol.*, xxvi, 142, 1896.

Orange (National Aniline and Chemical Company); Blue Base (Hudson River Aniline Color Works); Dandelion Brand Butter Color (Wells, Richardson and Company); and Annatto. These are water-insoluble compounds which are soluble in fat, fatty acids, alcohol, ether, chloroform, benzene and bile, as well as in solutions of the isolated bile salts. They were introduced, dissolved in fat or in lecithin emulsions of oil,³ either by feeding or by intravenous, subcutaneous or intraperitoneal injections. The dyes deposited in the fatty tissue and secreted milk of the experimental animals were easily detected by the color; those in the glandular, muscular, and nervous tissue, and in the fluids of the body—the blood, lymph and bile—were less easily determined. In all cases 2-gram portions of the tissue to be examined were minced, dried with anhydrous sodium sulphate and extracted with ether. The ether extracts were filtered, allowed to evaporate in white porcelain dishes, and the colors of the residues were noted. The blood and lymph were also dried with anhydrous sodium sulphate; the bile was similarly extracted with ether after being dried down with calcium oxide to form ether-insoluble compounds of the bile pigments.

DEPOSITION OF FAT-SOLUBLE DYES IN ANIMAL TISSUES.

With the exception of the meal worm, *Tenebrio molitor*,⁴ the infusoria (Staniewicz) and possibly the cow⁵ the adipose tissue has been found to be stained in those animals into which fat, stained with Sudan III, has been introduced. The animals investigated, the methods of introducing the stain and the results obtained, are summarized in the table on pages 74 and 75.

Although the time required to stain the adipose tissue of animals of different species has been noted only incidentally, it would seem from the results reported that it varies considerably. Riddle has observed that rabbits and turtles absorb Sudan III less rapidly than the fowl, in which the fatty tissue is colored after one or two days' feeding. The red stain appeared in the yolk of the eggs of hens,

³ This emulsion, supplied by Fairchild Bros. and Foster, consisted of 5 per cent lecithin, 45 per cent peanut oil and 50 per cent water.

⁴ Biedermann: *Arch. f. d. ges. Physiol.*, lxxii, p. 105, 1898.

⁵ S. H. and S. P. Gage: *Science*, xxviii, p. 494, 1908; *Anatomical Record*, iii, 1909.

and in the milk of rats after one feeding, whereas the cow observed by S. P. and S. H. Gage⁶ gave no evidence of Sudan absorption after four days of Sudan feeding.

EXPERIMENTAL. Feeding experiments with *Sudan III* were carried out with rats, cats, guinea pigs, pigeons, hens, frogs, a cow and a goat. The results were comparable with those of the earlier investigators. After a single feeding of deeply stained food, colored fat was found in the milk of cats and rats, and in the egg of the hen. Pigeons, after five days, showed a distinct pink coloration of the subcutaneous tissue through the skin; at autopsy, the fatty tissue was found to be distinctly stained. Three guinea pigs, to which were given two gelatin capsules, each containing 80 mgms. of Sudan III, every second day for four weeks, gave no evidence of stained tissue; two guinea pigs, given 2 cc. of stained oil every second day for three weeks, contained faintly pink adipose tissue. Frogs were fed for three weeks during the hibernating period with meat liberally mixed with stained oil; throughout the experiment they were kept in a room at 20°C. In no case did the fatty tissue of these become stained. The cow secreted no stained milk even after seven successive feedings of 7.5 grams of Sudan dissolved in oil; whereas the milk of the goat was faintly, but distinctly, pink after one feeding of Sudan-stained food.

It will be observed that, in general, those animals (rats, cats and fowls) which absorb fat readily, give evidence of Sudan-stained fat in less time than those, like the guinea pig and cow, in which fat forms a smaller factor in the diet.

Biebrich Scarlet, which resembles Sudan III in its solubilities, and is not affected by dilute solutions of acids and alkalies, was fed to pigeons, rats and cats, with results comparable with those obtained with Sudan III. The subcutaneous tissue was colored pink.

Feeding experiments with *indophenol-blue* were unsuccessful. This dye, unaffected by dilute alkalies, changes to pale yellowish green when treated with dilute hydrochloric acid. This color change in the stomach made it impossible to detect the dye absorbed. In rabbits and pigeons after subcutaneous injections of oil emulsions colored with the blue dye no fatty tissue was found

⁶ *Anatomical Record*, iii, 1909.

Animals stained with fat-soluble dyes.

INVESTIGATOR	ANIMAL	DYE	METHOD OF INTRODUCING DYE	TISSUES AFFECTED	REMARKS
Daddi ('96)	Rabbits Guinea Pigs	Sudan III Sudan III	Feeding stained oil Feeding stained oil	Subcutaneous Subcutaneous	.
Biedermann ('98)	Pigeons <i>Tenebrio molitor</i>	Sudan III Sudan III	Feeding stained oil Feeding stained oil	Subcutaneous None	Body fat uncolored.
Sitowski ('05)	Caterpillar	Alkanna Sudan III	Feeding stained oil Feeding stained	None Body and Eggs	Body fat uncolored.
Hofbauer ('05)	Guinea Pigs	Sudan III	Feeding stained fat wool	Adipose tissue	
Riddle ('08)	Fowl	Sudan III	Stained food	Adipose tissue and eggs	
	Rabbits	Sudan III	Stained food	Adipose tissue	Rabbits become stained less readily than fowl.
	Turtles	Sudan III	Stained food	Eggs	Turtles become stained less readily than fowl.
Riddle ('10)	Rabbits	Sudan III	{ Parenteral injection Intravenous injection	Adipose tissue Adipose tissue	
S. P. and S. H. Gage ('08)	Fowl	Sudan III	Stained food	Adipose tissue and eggs	Adipose tissue of chicks from stained egg is colored.

	Rats	Sudan III	Stained food	Adipose tissue	Milk colored; young born not colored.
S. P. and S. H. Gage ('09)	Guinea Pig	Sudan III	On carrots	No examina- tion	Young not colored.
	Cow	Sudan III	Stained food	No examina- tion	Milk not colored.
Staniewicz ('10)	Infusoria	Sudan III	Feeding stained fat	None	Body fat uncolored.
Mann*	Cats	Scharlach Rot	Feeding stained oil	Adipose tissue	
Mann*	Cats	Alkanna	Feeding stained oil	Adipose tissue	

*These data were sent to the writer by Prof. Gustav Mann, and it is with his permission that they are given here.

stained. The blood of rabbits taken from two to six hours after intravenous injections of indophenol-blue dissolved in oil emulsion yielded pink residues on extraction.

These results point to the reduction of the indophenol-blue to indophenol by the tissues. The presence of active reductases in the various tissues of the animal body has been observed by Ehrlich,⁷ Herter,⁸ Harris⁹ and others. Heffter¹⁰ reports that the liver is particularly rich in this enzyme, a fact which was further demonstrated by us as follows:

Ground liver tissue, to which oil stained with indophenol-blue had been added, was allowed to autolyze in the presence of toluene at a temperature of 30°C. After twenty-four hours, the mixture had lost its blue color and had become pink; the addition of hydrogen peroxide brought back the blue color. No change in color took place in a control experiment, carried out with boiled liver tissue under identical conditions.

The localization of fat-soluble dyes in the tissues.

Analysis of the various tissues of the animal body shows that the largest quantity of fat (ether extract) is found in the subcutaneous tissue, the fatty tissue of the abdominal cavity and the bone marrow; however, the muscular, glandular and nervous tissues contain estimable amounts. It is reasonable to suppose, therefore, that animals containing Sudan-stained adipose tissue would likewise have stained fat in the other fat-bearing tissues, especially since this dye readily reveals the presence of fat in histological sections of these tissues. The only investigators who even suggest that the fat of other than the adipose tissues may not be colored are Mann¹¹ and S. P. and S. H. Gage.¹² The basis for Mann's statement that "animals fed on oil colored with Sudan III show only the adipose tissue stained" is not clear. S. P. and S. H. Gage failed to find the stain in the nerve fibres of the chicks developed from the Sudan-stained eggs, although the adipose tissues of these were distinctly colored.

⁷ *Das Sauerstoffbedürfniss des Organismus*, Berlin, 1885.

⁸ *Amer. Journ. of Physiol.*, xii, pp. 207, 457, 1904-5.

⁹ *Bio-chem. Journ.*, v, p. 143, 1911.

¹⁰ *Medizinisch naturwissenschaftliches Archiv*, i, p. 81, 1907-8.

¹¹ *Physiological Histology*, 1902, pp. 36-7.

¹² *Science*, xxviii, p. 494, 1908.

Bondi and Neumann¹³ found that the bone marrow and livers of rabbits were distinctly blue after the injection of an emulsion of fat, stained with indophenol, and that the Kupfer cells of the livers of rabbits became distinctly pink after the injection into the circulation of an oil emulsion stained with Scharlach Rot. The animals were killed a few hours after the injection; the adipose tissue had not become stained in this short time, and the fact that the liver cells contained the color of the dye injected cannot be taken as proof that these cells store fat. The results of subsequent experiments in this investigation pertaining to the mode of elimination of fat-soluble dyes, to which reference will be made later, have thrown some light upon this point, and make it evident that these observations of Bondi and Neumann may be otherwise interpreted.

EXPERIMENTAL. In order to ascertain whether stained fat, other than that in the distinctly adipose tissue, is present in the bodies of animals into which fat-soluble dyes have been introduced, 2-gram portions of the tissues to be examined were freed, as far as possible, from extraneous fat and connective tissue, finely divided, dried and extracted with ether in accordance with the method already described. The dyes were administered dissolved in olive oil or in lecithin emulsion of peanut oil. The results are summarized in the table on pages 78 and 79.

DISCUSSION. Negative results were always obtained from nervous and renal tissues; from muscle when it was freed from connective tissue or extraneous fat as in starvation; and in general from liver tissue. Livers however from which blood had not been removed by perfusion or bleeding sometimes showed traces of the dye. In two cases the livers from rats which had been fed on a diet containing 75 per cent of deeply stained lard, yielded considerable quantities of the dye. These livers were distinctly pink, owing undoubtedly to the storage of the absorbed fat in the liver cells. Microscopic examinations of frozen sections, however, failed to disclose the dyes, even when chemical isolation demonstrated their presence.

The explanation of these results is not clear. It may be that the form of the fat in the nervous, muscular and glandular tissues

¹³ *Zentralbl. f. Biochem. u. Biophysik*, x, p. 1453, 1910.

Tissues stained with fat-soluble dyes.

+ means present; - means not detected; ? means trace.

ANIMAL	DYE	MODE OF INTRODUCTION	RESIDUE FROM ETHER EXTRACT OF					REMARKS	
			Brain	Kidney	Marrow	Muscle	Liver		Adipose tissue
Cat A.....	Sudan	Feeding	-			?	-	+	
Kitten.....	Sudan	Intra-peri- toneal in- jection							
Cat B.....	Sudan	Intra-peri- toneal in- jection	-			-	-	+	
Cat C.....	Sudan	Feeding	-	-	+	?	-	+	
Cat D.....	Sudan	Feeding	-	-	+	?	-	+	
Pigeon N.....	Annatto	Feeding	-	-	-	-	-	-	Died from starvation.
Pigeon A.....	Sudan	Feeding	-	-	+	-	+	-	Died from starvation.
Pigeon B.....	Sudan	Feeding	-	-	+	-	-	-	Died from starvation.
Pigeon D.....	Sudan	Feeding	-	-	+	-	-	+	Died as the result of phosphorus poisoning.
Hen.....	Sudan	Feeding	-						
Rat XI, 24.....	Sudan	Feeding	-		+	-	-	+	
Rat XI, 26.....	Sudan	Feeding	-	-	+	-	-	+	
Rat XI, 27.....	Sudan	Feeding	-			-	-	+	
Rabbit XI, 28.....	Sudan	Feeding				-	-	+	
Rabbit I, 5.....	Sudan	Feeding		-		-	-	+	
Rabbit 9.....	Sudan	Feeding				-	-	+	
Pigeon I.....	Indophenol	Feeding	-				-	-	

Died from starvation.
Died from starvation.
Died from starvation.
Died as the result of phosphorus poisoning.

Rat C.....	Sudan	Feeding	-	-	-	-	Starved 60 hours.
Rat D.....	Sudan	Feeding	-	-	-	+	Starved 24 hours.
Rat E.....	Sudan	Feeding	-	-	-	-	Starved 60 hours.
Rat 5.....	Sudan	Feeding	-	-	+	+	Starved 48 hours.
Rat XI, 12.....	Sudan	Feeding	-	-	+	+	Food contained 75 per cent stained fat.
Rat XII, 9.....	Sudan	Feeding	-	-	+	+	Food contained 75 per cent stained fat.
Rat XI, 5.....	Sudan	Feeding	-	-	+	+	Died as the result of phosphorus poisoning. Liver dark red.
Rat II, 10.....	Biebrich Scarlet	Feeding	-	-	-	+	

of the body is quite different from that in the adipose tissue—that it is held in some loose chemical combination which is no longer capable of taking up the stain. The present methods of fat extraction and staining may result in a disintegration of this complex molecule. MacLean and Williams¹⁴ have advanced the theory that the fat removed by extraction from animal tissues does not represent the form in which the fat exists in these tissues, and that the fat is made evident as the result of certain post-mortem changes by which the compound is broken up and the fat liberated. Leathes¹⁵ and Abderhalden and Brahm¹⁶ have suggested that the fat of the active tissues differs from that of the storage tissue. In the present investigation it was found that the isolated ether-soluble substances of the brain can take up the stain. This observation, together with the fact that the nervous tissue of Sudan-stained animals is always free from the dye, even when the embryonic fat contained an abundance, as was demonstrated by S. P. and S. H. Gage¹⁷ in chicks developed from the stained eggs, adds weight to the theory outlined above.

An explanation of the fact that in a large number of the experiments the liver tissue was found to be free from the dye, is afforded by the observation that *the fat-soluble dyes are more soluble in bile than in fat; and when these dyes are introduced into the body in solution in the fat they are eliminated in the bile.* Added evidence in favor of this explanation is found in the fact that the fat complex in the liver is not incapable of holding the dye in combination. This is shown by the following experiment:

A solution of Sudan III in bile was injected, under pressure, into the common bile duct of a rabbit. After 20 cc. had been forced in, the liver was removed, perfused with physiological saline solution, comminuted, washed in cold running water for twenty-four hours and filtered; the residue, which was distinctly pink, was washed until the filtrate gave no test for bile salts with Pettenkofer's reaction; ether extracts of the dried residue were distinctly pink. There could be no doubt that the fat had absorbed the stain.

CONCLUSIONS. Stained fats, introduced into the animal body intraperitoneally, intravenously, subcutaneously or by absorption

¹⁴ *Bio-chem. Journ.*, iv, p. 455, 1909.

¹⁵ *Problems in Animal Metabolism*, 1906, p. 72.

¹⁶ *Zeitschr. f. physiol. chem.*, lxx, p. 330, 1910.

¹⁷ *Science*, xxviii, p. 494, 1908.

from the alimentary tract, are laid down in the adipose tissue and marrow.

The renal and nervous tissues are free from the stain, even when the fatty tissue is deeply colored; muscle tissue, when freed from fat, as in extreme starvation, contains no stained fat; the dye is found in the liver only when the blood contains an abundance, as in starvation, or when the animal has been fed food containing a large amount of stained fat a few hours before the examination.

Liver fat, *in situ*, is capable of taking up the stain.

Indophenol-blue is reduced in the body; this reduction takes place, in part, in the liver; hence adipose tissue is not stained with this dye.

AVAILABILITY OF STAINED FAT IN METABOLISM.

Riddle¹⁸ has suggested that adipose tissue stained with Sudan III is less available to the organism than unstained adipose tissue. Inasmuch as the dye enters into no chemical union with the fat, but is merely dissolved therein,¹⁹ it does not seem probable that the Sudan III can so change the nature of the fat that it cannot be used as effectively by the organism as unstained fat. An indifferent material, like Sudan III, might be toxic, or might form toxic combinations in the body, and thus affect organs dealing with fat combustion; but that the fat itself is rendered unavailable scarcely seems tenable. The non-toxicity of Sudan III has been shown by feeding animals over long periods of time without apparent deleterious results.

EXPERIMENTAL. In order to determine if Sudan-stained fat is less available to the organism, starvation experiments were carried out with Sudan-stained rats and pigeons; comparable experiments were conducted with normal animals.

1. *Pigeon B.* Fed with pulverized dog biscuit, lard deeply stained with Sudan III, and cracked corn for three weeks before the beginning of the fasting period. Subcutaneous tissue became pink. Weight of pigeon at beginning of fast was 297 grams. Death in ten days. It had lost 116.5 grams, 39 per cent of its initial weight; all visible fat had disappeared. No pink color was to be seen. A slight trace of Sudan III was found in

¹⁸ *Journ. of Exp. Zool.*, viii, p. 163, 1910.

¹⁹ Michaelis: *Virchow's Archiv.*, clx, p. 263, 1901.

ether extract of the tail gland, bone marrow and liver; the muscle, kidney and brain contained no trace of the dye.

2. *Pigeon C.* Preliminary feeding same as B. Subcutaneous tissue became noticeably pink. Weight at beginning of fast 294 grams. Death in eleven days. Loss of weight 165 grams, 56 per cent. No visible fat remained; tissues showed no pink color; ether extract of liver and bone marrow slightly pink; of muscle, kidney and brain, colorless.

3. *Pigeon D.* Fed Sudan-stained food as in B and C. After sixteen days of fasting this animal died. It had lost 41 per cent of its initial weight. All visible fat and stain had disappeared from the body. The ether extract of the bone marrow was slightly pink; that from the liver and muscle showed no pink color.

4. *Pigeon E.* A normal well-fed bird, was starved for sixteen days, during which it lost 229 grams or 54 per cent of its initial weight. All visible fat had disappeared from the body.

5. *Pigeon F.* A well-fed normal bird which died fifteen days after the fasting period began. The loss in weight was 144 grams or 45 per cent of its initial weight. All visible fat had disappeared.

It should be noted that Pigeons B and C were kept during November in an unheated room with the windows open. This doubtless explains their earlier death as compared with pigeons D, E and F which were kept at about 20°C. In every case, however, the fatty tissue had entirely disappeared from the body.

Experiments with rats gave similar results.

6. *Rat A.* Fed with ground dog biscuit mixed with lard deeply stained with Sudan III for seven days. Fasting period, three days. Loss in weight, 42 grams, 42 per cent of initial weight. The body was free from all traces of visible fat and stain.

7. *Rat C.* Preliminary feeding period, same as A. Fasting period approximately sixty hours. All visible fat disappeared from the body. The ether extract of the brain, liver, muscle, kidney and subcutaneous tissue left no pink residue.

8. *Rat B.* A normal well-fed rat, which died after a 60 hours' fast. The loss in weight was 40 grams or 22 per cent of its initial weight. The body was free from all visible fat.

9. *Control experiment.* *Rat D.* Was fed on Sudan-stained food as in the previous experiments, for seven days. The subcutaneous tissue, omentum and fatty tissue about the kidneys were deeply pink.

The result of the control experiment affords evidence that the adipose tissue of the experimental animals was similarly stained at the beginning of the fasting periods. The further observation was made that rats, and in some cases rabbits, stained as the result of feeding with deeply stained, fat-rich food, excreted urines which

were distinctly pink; such urines were found to contain both fat and Sudan III.

In two experiments Sudan-stained pigeons were fed with unstained foods after long fasting periods—other pigeons, fasting the same length of time and under similar conditions, had died. At autopsy, the fatty tissue of these was found to be unstained.

10. Pigeon G. Fed with Sudan-stained fat, described in protocols 1-3, starved thirteen days; loss of weight, 104 grams or 29 per cent. It was re-fed and examined some months later. All trace of Sudan had disappeared; the ether extract of the tissues left no pink residue.

11. Pigeon A. Previously fed with Sudan-stained food; starved eleven days; loss of weight, 104 grams or 32 per cent. It was re-fed until it had gained 16 grams. Upon examination, no stained tissue was found. Ether extract of subcutaneous fat, tail gland and omentum showed no pink color.

DISCUSSION. The results of these trials are not in agreement with those reported by Riddle. Sudan-stained pigeons and rats died in no less time than the unstained control animals. In both cases the visible fat had entirely disappeared, and, in the stained animals, the dye as well. Those animals which were fed after long fasting periods until there was a marked increase in body weight, contained no trace of the former Sudan-stained fatty tissue. One must conclude from these results that stained adipose tissue is no less available to the organism than the non-Sudan-stained fat and that it is used quite as readily and completely.

The disparity between our results and those of Riddle is difficult to explain. His observations that chicks fed on stained food developed more slowly than normal chicks and that hens ceased to lay after considerable quantities of the dye had been ingested may have resulted from other causes than the ingestion of the dye. It is conceivable that the apparent failure of starving stained animals in his experiments to utilize their fatty tissues as do normal animals was the result of impurities in the dye fed. Mann²⁰ has observed that Scharlach Rot given to half grown kittens in large doses causes vomiting. We gave large doses of Sudan III, put up by an American manufacturer, to two cats. These died within a comparatively short time apparently from the effect of some impurity in the dye. Other cats, given equally large doses of the Kahlbaum dye, experienced no ill effects. Riddle's deductions from

²⁰ Personal communication.

his second series of fasting experiments that stained animals underwent a greater percentage loss of weight during starvation than do unstained are unconvincing by reason of the fact that an important part of his weighing records was lost.

THE FATE OF FAT-SOLUBLE DYES IN THE ORGANISM.

The observations cited above have shown that Sudan III, deposited in the tissues as the result of adding the dye to the food, disappears completely during starvation. Experiments upon cats and rats gave no reasons for thinking that this disappearance is due to elimination of the dye by the kidneys. The fact that the excreta of starving Sudan-stained pigeons contained the dye and the observation that the dye was present in the gall bladders of Sudan-stained animals subjected to starvation or poisoning with phosphorus or phlorhizin turned our attention first to the elimination of fat-soluble dyes by way of the bile. It is well-known that the bile is the normal path of elimination of many substances. From the work of Abel and Rountree²¹ on phenoltetrachlorophthalein the assumption seems justifiable that substances which leave the body exclusively by way of the bile must be insoluble in water and soluble in bile or substances contained therein.

Two preliminary experiments were made upon cats, previously fed with Sudan III and starved for four days preceding the experiment. The bile, collected as it was secreted by the liver, and the blood yielded pink ether extracts, while those obtained from the liver tissue, washed free of blood and bile, were colorless. These results pointed to a transport of Sudan-stained fat to the liver with subsequent storage of the fat in the liver and elimination of Sudan III in the bile.

The elimination of fat-soluble dyes under normal conditions.

The dyes, dissolved in lecithin-oil-emulsion, were introduced into the circulations of cats, dogs and rabbits by injections into the femoral veins. In each case the urine contained in the bladders, as well as the liver tissue and bile, was examined for the injected stain. The approximate time of the appearance of the dye in the

²¹ *Journ. of Pharmacol. and Exp. Therapeutics*, i, p. 231, 1909.

bile after its introduction into the blood stream was incidentally noted.

The results of the experiments are summarized in the following table:

The excretion of fat-soluble dyes introduced dissolved in fat.

ANIMAL	DYE	COLOR OF DYE	RESIDUE FROM ETHER EXTRACT OF BILE	TIME OF APPEARANCE OF DYE IN BILE	DYE IN URINE	DYE IN LIVER
				minutes		
Cat II, 8.....	Indophenol	Blue	Blue	30†		None
Dog II, 14....	Sudan	Red	Pink	30	None	Present
Dog II, 16....	Sudan III	Red	Pink	20	None	None
Cat II, 21....	Indophenol	Blue	Blue		None	Present*
Dog II, 21....	Sudan III	Red	Pink	60†		
Cat II, 22....	Oil Green	Green	Green	30	None	None†
Cat II, 23....	Oil Green	Green	Green	90	None	None
Rabbit II, 28.	Biebrich Scar- let	Red	Pink	60	None	Present
Dog III, 21....	Sudan III	Red	Pink	60	None	None
Cat III, 24....	Sudan III	Red	Pink	75	None	None
Cat IV, 21....	Butter Color	Yellow	Pink	55	None	None
Cat V, 2.....	Oil Orange	Orange	Yellow- red	90	None	None
Cat V, 6.....	Blue Base	Blue	Pink	50	None	Present‡
Cat V, 15....	Butter Color	Yellow	Pink		None	

* The animal died fifteen minutes after the second injection of dye.

† The animal died one-half hour after the injection.

‡ The addition of dilute hydrochloric acid to the liver tissue resulted in a blue color. The animal died four hours after the injection.

In a number of the experiments the residues from the ether extracts of the excreted bile were examined for fat. The ethereal filtrates were allowed to evaporate from watch glasses; the residues were heated gently; no melting of the material took place and no grease spot was formed on soft tissue paper by this residue. The dyes were excreted dissolved in bile and not in combination with fat.

In some cases, the color of the residues from the ether extracts of the bile was not precisely like that of the dyes injected. This change in color is the result of the passage of the dyes through the body where they are brought in contact with hydroxylions. The action of dilute alkalies on the dyes outside the body causes a similar change.

The residues from the ether extracts of the liver tissue were not always colored; when the animals were killed some time after the injection of the stain, or when only a small amount had been introduced, the liver was found to be free from the dye. The urines were consistently free from stain.

It is obvious from these results that *fat-soluble dyes, when introduced into the circulation in solution in fat, become separated from the fat and are eliminated in the bile.*

Absorption of fat-soluble dyes into the portal circulation.

The next experiments were designed to determine the rôles played by the bile and by the fat in the absorption of fat-soluble dyes from the intestine.

22. Dog: 25 kgm. Fed at 7.20 a.m. Two hours later the animal was anaesthetized, and a cannula was inserted in the thoracic duct. Twenty cubic centimeters of Sudan-stained oil were injected into the duodenum at 11.15 a.m., followed by 1 gram of desiccated ox bile in solution. At 12.15 the lymph was intensely pink. At 2.00 p.m., a cannula was inserted in the bile duct. The animal was killed by bleeding at 5.30 p.m. Two-gram portions of the liver tissue, 10 cc. samples of the blood, 50 cc. of the lymph and from 2 to 4 cc. of the bile were examined. The ether residues from the dried lymph and bile were distinctly pink, while those from the blood and liver showed no trace of the dye.

23. Cat, full-grown. Fed at 8.15 a.m., was anaesthetized at 9.30 a.m. A cannula was inserted in the thoracic duct at 10.45 a.m.; after 10 cc. of Sudan-stained emulsified oil had been injected into the duodenum, a cannula was placed in the common bile duct and the bile collected therefrom. The lymph flowed freely, and at 2.30 p.m. it was distinctly pink in color. The ether residues from the lymph and bile were distinctly pink. The blood (10 cc.) taken at 5.00 p.m. yielded no pink residue. The liver was also free from the dye.

These experiments show clearly that although Sudan III, introduced with fat into the intestine, is absorbed by the lacteals and appears in the thoracic lymph, it is still absorbed and eliminated in the bile, under conditions which preclude the entrance of the lymph into the blood. In the latter case, neither the blood of the general circulation nor the liver tissue is stained. This behavior is explained in part by the observation that the dyes studied are more soluble in bile than in fat and by the results of the following experiments which show that the dyes may be absorbed from the intestine into the portal circulation in solution in bile.

Fasting animals were anaesthetized, a cannula inserted into the bile duct and bile solutions of the various dyes used in the earlier experiments were injected into the small intestine. The results are summarized below.

The excretion of fat-soluble dyes absorbed from alimentary tract, dissolved in bile.

ANIMAL	DYE	COLOR OF DYE	RESIDUE	DYE IN LIVER	DYE IN BLOOD	DYE IN URINE
			FROM ETHER EXTRACT OF BILE			
Cat II, 8.....	Sudan III	Red	Pink	None	None	None
Cat II, 8.....	Blue Base	Blue	Blue	None	None	None
Cat III, 10.....	Indophenol	Blue	Blue	None	None	None
Cat III, 10.....	Oil Green	Green	Brown pink	None	None	None
Rabbit III, 20...	Biebrich-Scarlet	Red	Pink		None	None
Cat V, 29.....	Oil Yellow	Orange	Yellow pink	None		

The presence of the dye in the bile in these experiments and its absence from the blood of the general circulation show clearly that it is absorbed with the bile by the portal circulation and eliminated with the bile by the liver. That none of the dye entered into the general circulation is evidenced by the fact that the blood of the animals examined—five out of six—gave no indication of even traces of the dye when tested by a method capable of detecting 0.00001 gram of Sudan III in 10 cc.

The two following experiments show that when bile is not present with the dye in the intestine, no absorption of the dye in the portal circulation occurs.

Stained fat was introduced into a loop of the upper intestine after this had been washed out with physiological saline solution to remove all traces of the adherent bile. The bile excreted under these conditions was free from the stain, although in one case (cf. protocol 24) the thoracic lymph showed that a slight amount of fat absorption had taken place; in the other experiment (cf. protocol 25) both bile and lymph contained no dye until after the introduction of a bile solution into the intestinal loop, when the excreted bile was found to contain Sudan III, although the lymph was still colorless.

24. *Dog: 20 kgm.* Narcotized with morphine and ether. A temporary lymph cannula was inserted at 10.00 a.m., and a bile cannula at 10.45 a.m. A 12-inch loop of the intestine was tied off just below the pylorus and washed out with physiological saline solution, at body temperature, until the washings were clear. Sudan-stained oil, together with a solution of 0.1 per cent HCl, introduced to increase the pancreatic secretion, were injected into the intestinal loop at 11.00 a.m. Bile, collected at 12.00 m., 2.00 p.m. and 3.30 p.m., when dried and extracted, left no pink residues. Seventy cc. of lymph, collected between 2.00 and 3.30 p.m., contained a small amount of Sudan III; the ether extract of dried blood was unstained.

25. *Dog: 8 kgm.* Anaesthetized with morphine and ether at 9.45 a.m. The insertion of the temporary lymph cannula immediately preceded that of the bile cannula. The intestine was ligated just below the pylorus and 14 inches below it. This loop was washed out with physiological saline solution until the washings were clear. Approximately 10 cc. of Sudan-stained emulsified oil, together with 10 cc. of 0.1 per cent HCl were introduced into this loop. The bile collected at 3.30 p.m. left no pink residue; the lymph also was free from dye. At 3.30 p.m., 10 cc. of a solution of desiccated ox bile were injected into the intestinal loop. The bile collected at 7.30 p.m., 3.5 cc., showed the presence of the dye, while the lymph taken at this time, 25 cc., left no pink color when extracted.

The elimination of the dye in the bile during fat absorption, under conditions where the stained fat was prevented from entering the general circulation, was undoubtedly due to the migration of the dye from the fat to the bile in the intestine and its subsequent absorption. There is no reason to believe that the dye in the excreted bile was the result of absorption of stained fat into the portal circulation. Had such been the case, the dye would have been present in the excreted bile in experiment 24, as well as in the lymph.

The time required for the absorption and deposition of fat, studied by means of fat-soluble dyes.

The fact that fat-soluble dyes are eliminated in the bile explains some hitherto inexplicable phenomena observed in the work with Sudan III. Earlier in this investigation an attempt was made to determine the length of time required to lay down the fat absorbed from the alimentary tract. Stained fat was fed to rabbits and cats; and samples of blood, taken from the ear veins of the rabbits and the jugular veins of the cats, were examined for the circulating dye. The stain was still found to be present in the blood of rabbits

one week after the last stained feeding; and the blood of the cats, tested from four to five weeks after the last Sudan-feeding, left distinctly pink residues. Oil emulsions stained with Sudan III and Biebrich Scarlet gave similar results when injected into the circulation of rabbits. The blood of these was found to contain the dye three weeks after the last injection.

These observations find their explanation in the fact that dye, absorbed from the intestine into the lymph with the fat and into the portal blood with the bile, again enters into the intestine with the bile. Thus a closed circulation of the dye is established and it is possible that the blood of a once Sudan-stained animal may become quite free from the stained fat only after long periods under normal conditions of feeding. Animals examined months after the Sudan-stained feeding had ceased contained deeply stained fatty tissue.

The time required for the elimination of circulating fat-soluble dyes.

An attempt was made to ascertain (in cats and dogs) the length of time necessary for the separation of the dye from the circulating fat and its elimination through the bile. Emulsions of stained fat, in amounts varying from 1 to 10 cc., were injected directly into the circulation; cannulae were placed in the common bile ducts and samples of blood and bile were taken every two or three hours. In order to facilitate the flow of bile, solutions of desiccated ox bile were injected into the upper intestine. The bile, blood, and liver tissue after it had been washed free from blood, so far as possible, were examined for the stain.

Nine and one-half hours was the longest period during which observations were made in any experiment; and although in that instance only 1 cc. of the stained emulsion was injected, both blood and bile, collected at the end of this time, showed that a considerable quantity of stained fat was still in circulation. In those cases in which the experiments continued over a comparatively long time, or when a small amount of the stained fat had been introduced, the liver was free from the stain. The liver evidently does not store up stained fat; the dye becomes separated from the fat as the stained fat comes in contact with the bile in the liver cells.

DISCUSSION. Fat-soluble dyes introduced into the body in solution in fat are secreted in the bile. These dyes may enter the

body from the alimentary tract in two ways: (1) in the lymph, in solution in fat; (2) through the portal circulation, dissolved in reabsorbed bile. When the dyes are absorbed dissolved in bile, they apparently do not pass beyond the liver, but are speedily reexcreted into the gut, and do not enter the general circulation unless fat is present in the intestine. The blood of Sudan-stained animals, under normal conditions of feeding, is never free from the fat stain. The dye put out in the biliary secretion is reabsorbed in the digesting fat, and a continuous circulation from gut to blood and return is established. The elimination of the stain from the circulation, when all possibility of reabsorption is removed, takes place slowly. The stained fat was found in the blood of a cat nine and one-half hours after it had been injected into the femoral vein.

FAT TRANSPORT IN STARVATION AND PATHOLOGICAL CONDITIONS:
PHOSPHORUS AND PHLORHIZIN POISONING.

We have attempted to follow the migrations of Sudan-stained fats under conditions in which a transport of fat is well-known to occur, namely, in starvation and after poisoning with phosphorus or phlorhizin. The experimental animals were fed in advance for a period of three to five weeks on Sudan-stained food. Phosphorus was administered subcutaneously, dissolved in oil; phlorhizin similarly in solution in sodium carbonate. Other details of selected protocols are summarized in tabular form:

Sudan III in pathological fat transport and starvation.

	ANIMAL	DURATION OF EXPERI- MENT	FAT CONTENT OF LIVER	STAIN IN	
				Bile	Blood
Starvation	Cat I, 13.....	5	56.0	Present	Present
	Cat I, 18.....	5	33.5	Present	Present
	Guinea pig....	1	7.9		Present
Phosphorus poisoning	Cat XI, 23....	9	64.3	Present	Present
	Hen X, 20.....	4	59.0	Present	
	Hen XI, 29....	19	40.5	Present	
Phlorhizin poisoning	Cat XII, 6....	12	15.8	Present	Present
	Cat XI, 19....	7	11.1	Present	Present

Neither in the foregoing nor in numerous other comparable experiments in which a transport of fat (fatty infiltration) was induced, was any evidence obtainable of dye in the extracts of the liver tissue or in frozen sections thereof. The constant finding of the Sudan III in both the blood and bile makes it evident that the dye migrates from the stained adipose tissue and is brought to the liver where it is eliminated in the bile. The observations give an additional indication that the fatty livers in these pathological conditions are produced by infiltration of fat; for it is difficult to believe that, if the high content of liver fat had been obtained by a degeneration process in the hepatic tissue, such an accumulation of dye in the bile would have taken place.

FAT TRANSPORT TO THE EMBRYO.

The question of the origin of foetal fat has been much debated;²² It involves the broader problem of the passage of substances through the placental barrier. S. H. and S. P. Gage ('08) failed to find the adipose tissues of the young stained, when stained fats were fed to pregnant mothers. Hofbauer ('05) believed that he found particles of dye in the foetal blood and assumed that they had become separated from fat metabolized by the embryo. His method—microscopic examination—is scarcely adapted to determine this point, however.

Numerous experiments in which we have fed rats and cats with Sudan-stained food or Biebrich Scarlet throughout the period of gestation have uniformly shown *an absence of the dye in the foetus or the newly-born young*. Two illustrative protocols, selected from many similar ones, will suffice to show our method of investigation.

Rat C. Sudan-feeding was begun sixteen days before the young were born. The alimentary tract was removed from one of them soon after birth. Its contents were distinctly pink (from mothers' milk). The ether extract of the entire residual body was uncolored. Subsequent examination of the mother showed deeply stained adipose tissue.

²² Cf. Ahlfeld: *Centralbl. f. Gynaekol.*, i, p. 265, 1877; Thiemich: *Centralbl. f. Physiol.*, xii, p. 850, 1898; *Jahrb. f. Kinderheilk.*, lxi, p. 174, 1905; Hofbauer, J.: *Arch. f. Gynaekol.*, lxxvii, p. 139, 1906; Oshima: *Zentralbl. f. Physiol.*, xxi, p. 297, 1907; Bondi: *Arch. f. Gynaekol.*, xciii, p. 189, 1911.

Cat B. Was fed 80 mgms. of Sudan III every second day for eighteen days prior to birth of kittens. Aside from the stomach contents there was no pink in the ether extract of tissues of the young examined soon after birth. The adipose tissue of the mother was deeply stained.

Although it is unlikely from such findings that stained fat can pass through the placenta, this is not necessarily conclusive evidence that the foetal fat has its origin in substances other than fat. The findings in the case of the alimentary epithelial tissues and glandular structures however add little likelihood to the transport or deposition of the fat in a non-stainable combination.

FAT TRANSPORT INTO MILK.

The precise relation of milk fat to food fat and the extent to which the latter can pass directly into the mammary secretion without first becoming a part of the body stores is not easily determined. S. H. and S. P. Gage ('09) found Sudan III in the milk of rats after prolonged feeding with the dye; this, however, is no proof of the immediate origin of the milk fat from the food, since the fat depots of the rats were also stained. In explanation of the observations that foreign food fats have more frequently been found secreted in the milk of smaller animals (goats, sheep, dogs, rats) than of cows, it has been suggested that the milk secretion is more directly dependent upon the food supply in the smaller species.²² However, the marked differences in the time required to stain the adipose tissue of guinea pigs and rabbits with Sudan III in comparison with cats and rats, suggests that the discrepancies noted above may bear some relation to the readiness with which the different animals absorb and store fat.

EXPERIMENTAL. We have investigated the appearance of Sudan III in the milk after feeding the dye both before and during the period of lactation. When animals, notably cats, have refused to eat stained fat, the dye has been administered in capsules either directly before or after a meal rich in fat. This fact is important for successful results. In the case of cats and rats the character of the milk was determined by examining the stomach contents of suckling young. Needless to say great care must be taken to

²² Cf. Lusk: *Science of Nutrition*, 1909, p. 237.

have the cages scrupulously free from stained food which might lead to erroneous conclusions.

It is scarcely necessary to repeat here the details of the many trials, since the methods are fairly obvious. Both Sudan III and Biebrich Scarlet were found to be secreted into the milk by rats; Sudan excretion was likewise observed in cats, guinea pigs and a goat. In the case of the goat, one gram of Sudan III dissolved in oil was added to the feed twice daily during six successive days.²⁴ The milk drawn nine hours after the first dose showed the presence of the dye, the tint increasing with the subsequent milkings. The guinea pigs received 2 cc. of stained olive oil every other day.

An important fact in this connection is the observation that the color disappears from the milk when the Sudan-feeding is discontinued, despite the persistence of the stain in the adipose tissues of the secreting animals. This was likewise true in experiments with Biebrich Scarlet.

The following protocol illustrates the transport of storage stained fat during starvation and the passage of the dye into the milk:

Rat 11. Was fed Sudan-stained food during the period of gestation. Soon after the birth of the young, April 30, the cage was cleaned and unstained food thenceforth employed. On May 14 the ether extract of milk found in the stomach of a suckling rat was uncolored. The mother was now starved two days. At the end of this time the milk in the stomach of another one of the young gave a faintly pink ether extract. The adipose tissue of the adult was found to be stained still. This experiment was duplicated with another mother.

Like S. H. and S. P. Gage, we have failed to induce the secretion of Sudan III in the milk of cows. A Holstein cow was given 7.5 grams, twice daily, dissolved in olive oil and added to the mash feed on three successive days, without positive results. In considering this we recall that the milk of the goat and guinea pig—animals in whose diet fat likewise plays a comparatively small rôle—was decidedly faint in color in comparison with the milk of cats and rats. Bearing in mind the necessity of fat for the transport of the dye an explanation at once suggests itself for the inequalities here observed.

²⁴ This experiment was conducted at the New York Agricultural Experiment Station in Geneva, through the kindness of Director W. H. Jordan.

SUMMARY.

Some of the fat-soluble dyes, introduced into the organism by various paths, are deposited in the adipose tissues and bone marrow. The renal and nervous tissues are free from the stain, even when the fatty tissues are deeply colored. Muscle probably does not take up the dye. It is seldom found in the liver, because the fat-soluble dyes, which are insoluble in water, dissolve readily in the bile and are excreted thereby into the intestine from which they can be reabsorbed.

The fat-soluble dyes may enter the organism from the alimentary tract through the lymphatics, in solution in fat; or by the portal circulation, dissolved in reabsorbed bile. They do not pass beyond the liver unless fat is present to transport them. Then they may be found in the blood, which is rarely free from the dye in a normally fed animal that has once been stained. A cycle between intestine, bile and blood becomes established. No elimination of the dyes occurs through the kidneys, except when an alimentary lipuria arises (in rabbits and rats).

Contrary to the assertion of others, the stained fat is no less available to the organism than the unstained.

In cases conducive to fat transport—in starvation, phosphorus- and phlorhizin-poisoning—stained fat migrates from the stained depots to the blood and the liver cells. Here the dye is separated and secreted into the bile; so that the liver, though having a high content of fat, may be free from the dye.

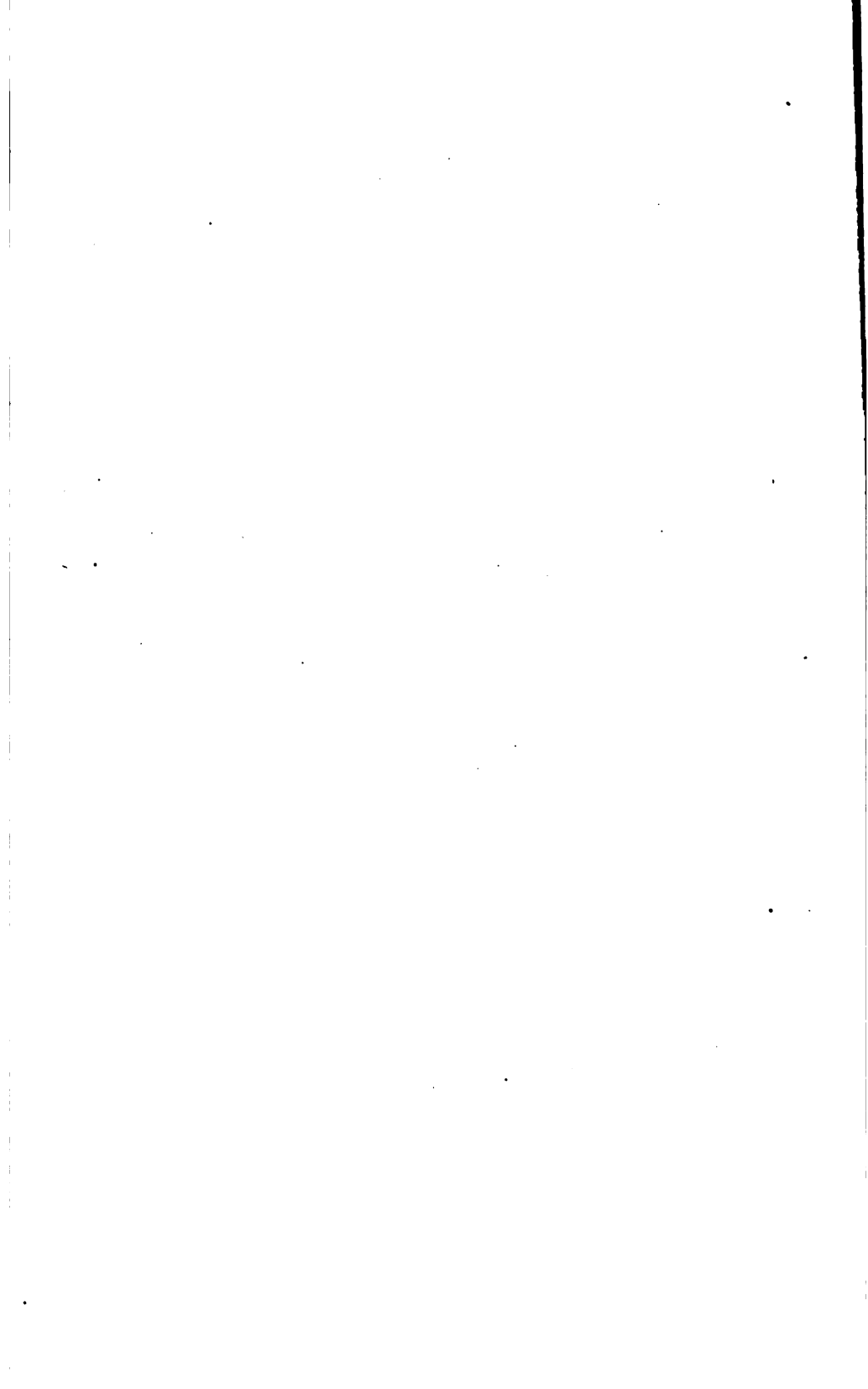
Stained fat does not traverse the placenta. The blood of the foetus and the fat of young born of Sudan-stained mothers is free from dye.

The excretion of Sudan III and Biebrich Scarlet in milk, when they are given with food fat, suggests that the latter may pass directly into the mammary secretion. With cats and rats the results are striking, but the dye excretion in milk ceases when the stained food is no longer fed. In guinea pigs and goats the secretion of dye in the milk is positive; in the cow it has not yet been demonstrated. The variation in the outcome in the different species may be due to variations in the relative abundance in the dietaries of fat necessary for the absorption and transport of the dye. This explanation is emphasized by the observation that those

animals (cats, rats, hens, pigeons) for which fat enters more largely into the diet, become stained more easily or speedily than animals which are accustomed to ingest relatively smaller amounts of fat.

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FAT-SOLUBLE DYES.

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SOME NEW COMPOUNDS OF THE CHOLINE TYPE. II.

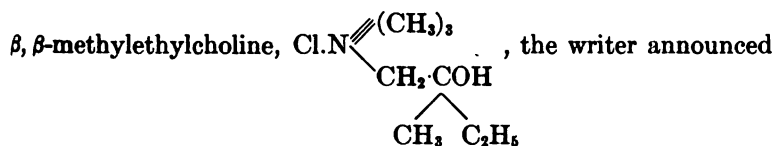
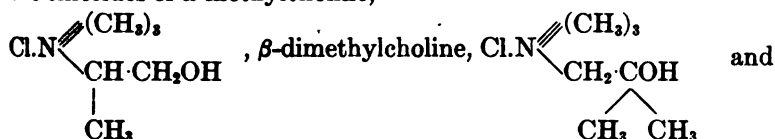
CERTAIN ACYL DERIVATIVES OF α -METHYLCHOLINE, " β -HOMO-CHOLINE" (β -METHYLCHOLINE) AND γ -HOMOCHOLINE.¹

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In a preliminary paper² under the same general title, describing the chlorides of α -methylcholine,



that certain acyl derivatives of α -methylcholine had also been prepared. The description of these compounds, however, was deferred at that time, pending the completion of certain minor details of investigation essential thereto. These experimental details have long since been completed but the published description of the compounds has been still further delayed by the demands of other work. Such description is here submitted together with that of several other acyl derivatives and certain of their salts.

A number of acyl derivatives of α -methylcholine chloride have been prepared. In all cases, except that of the acetyl derivative,

¹ Submitted, in abstract, to the International Congress of Applied Chemistry, New York, September, 1912.

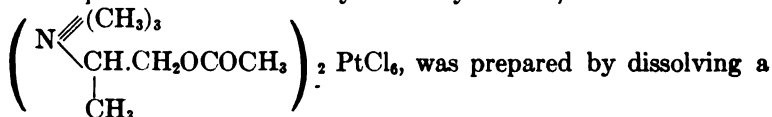
² This *Journal*, x, pp. 399-406.

the method of preparation was, in general, the same and consisted in heating the choline compound with a considerable excess of the acyl chloride, generally in a sealed tube at 100° . On pouring the reaction product into dry ether the acyl derivatives separated as oils or oily solids which were more or less difficult to purify according as the acylation had proceeded smoothly and completely or not. In all cases their preparation in only small quantities was attempted, primarily for pharmacological investigation, and any study of their general properties was accordingly restricted.

The acetyl derivative of α -methylcholine (acetyl- β -methylethoxytrimethylammonium chloride) $\text{Cl.N} \begin{array}{c} \text{(CH}_3\text{)}_3 \\ \diagup \\ \text{CH.CH}_2\text{OCOCH}_3 \\ | \\ \text{CH}_3 \end{array}$ was

prepared by mixing about 1 gram of the choline compound with a large excess of acetic anhydride in an acetylation flask and heating to gentle boiling for about two hours. After cooling, the reaction product was poured into a comparatively large volume of dry ether, whereupon the acetyl derivative separated as an oil or oily solid. This was repeatedly washed, by thorough stirring and decantation, with successive portions of dry ether to remove excess of acetic anhydride and the acetic acid. Finally the trace of ether remaining after decantation was driven off by gentle heating, the oily residue taken up in a little absolute alcohol and the alcoholic solution evaporated to dryness in a vacuum desiccator leaving a slightly oily, crystalline, hygroscopic, white solid. The final product was identified by means of the platinum and gold salts. In this connection, however, the platinum salts are considered the better because, as a rule, they are more definitely crystalline, less soluble, more stable, and more conveniently handled.

The platinum salt of acetyl- α -methylcholine,



portion of the chloride in a little absolute alcohol and precipitating with an alcoholic solution of chlorplatinic acid. A dense yellow crystalline precipitate resulted. The salt is insoluble in absolute

alcohol but soluble in water and in mixtures of alcohol and water. It melts³ with decomposition at about 222°–223° (corrected). Analysis for platinum by ignition resulted as follows:

0.1535 gram salt gave 0.0411 gram Pt.

	Calculated for $C_{12}H_{19}O_4N_2ClPt$:	Found:
Pt.....	26.70	26.77

The gold salt, $\left(N \begin{array}{l} \diagup (CH_3)_3 \\ \diagdown CH_2CH_2OCOCH_3 \\ | \\ CH_3 \end{array} \right) AuCl_4$, was prepared

in a manner similar to the platinum salt just described and also by adding a concentrated aqueous solution of chlorauric acid to a concentrated aqueous solution of the acetyl derivative. In each case the salt separated as a pale yellow, crystalline solid. Under the conditions outlined in a previous footnote it melts at 124°–125.5° (corrected). Analysis for gold by ignition in the two cases resulted as follows:

- I. 0.1459 gram salt gave 0.0574 gram Au.
II. 0.1373 gram salt gave 0.0540 gram Au.

	Calculated for $C_{12}H_{19}O_4NClAu$:	Found:
		I. II.
Au.....	39.48	39.34 39.33

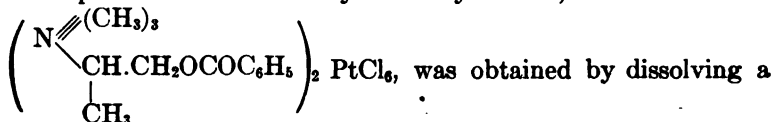
Benzoyl derivative of α -methylcholine chloride (benzoyl- β -methylethoxytrimethylammonium chloride), $Cl.N \begin{array}{l} \diagup (CH_3)_3 \\ \diagdown CH_2CH_2OCOC_6H_5 \\ | \\ CH_3 \end{array}$

This compound was prepared by heating the α -methylcholine chloride with a large excess of benzoyl chloride, in a flask with reflux condenser, to 100° (water bath) for about two hours. The reaction product poured into dry ether yielded an oily precipitate

³ All "melting points" here reported were determined by the usual capillary-tube method in a bath of sulphuric acid, using standardized Anschütz thermometers for which a correction for emergent stem was not considered necessary. The rate of heating in all cases was regulated so as to closely approximate 5° a minute within 20° to 30° of the melting point. As pointed out in Hygienic Laboratory Bulletin 70, and as corroborated in this and other work, the *decomposition* point may vary quite widely with comparatively slight variation in manipulation.

which was worked up in the same manner as the acetyl derivative except that the last traces of benzoyl chloride were removed by dissolving the oily precipitate in water and extracting several times with ether. The aqueous layer was then evaporated to dryness in a vacuum desiccator, containing concentrated sulphuric acid, yielding a white, crystalline, hygroscopic solid. The reaction was apparently neither so smooth nor so nearly quantitative as for the acetyl derivative.

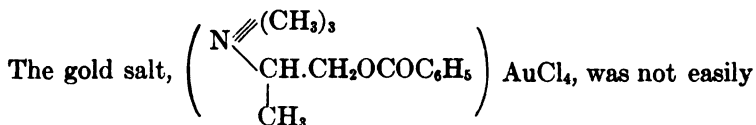
The platinum salt of benzoyl- α -methylcholine,



portion of the above described solid in a little cold water and precipitating with a cold aqueous solution of chlorplatinic acid. It appeared to be quite insoluble in cold water since careful concentration of the mother liquor caused no further separation. Heated in a capillary tube the salt begins to sinter definitely and to darken above 233° and decomposes with effervescence at 236.5° to 237.5° (corrected). Analysis for platinum by ignition resulted as follows:

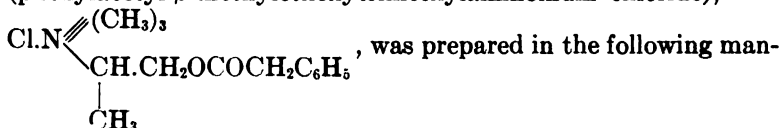
0.1178 gram salt gave 0.0270 gram Pt.

	Calculated for $\text{C}_{25}\text{H}_{40}\text{O}_4\text{N}_2\text{Cl}_6\text{Pt}$:	Found:
Pt.....	22.87	22.92



obtained, from the quantity available, in condition favorable for accurate analysis. It was precipitated from an aqueous solution of the benzoyl-choline derivative with concentrated aqueous chlorauric acid and separated as a pale yellow very viscous oil. It is quite soluble in alcohol and slightly soluble in hot water.

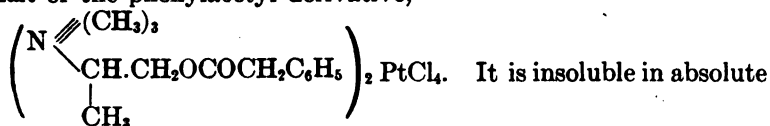
The phenylacetyl derivative of α -methylcholine chloride (phenylacetyl- β -methylethoxytrimethylammonium chloride),



ner: About 1 gram of α -methylcholine chloride was mixed in a Carius tube with a moderate excess of phenylacetylchloride; the tube was sealed, heated in boiling water for about five hours, allowed to stand in the cooling bath over night, and the product poured into dry ether. As in previous cases the acyl derivative separated in an oily condition.

In order to test the stability of the phenylacetyl group in the molecule a small portion of the oily product was taken up in cold water, extracted with ether, and the aqueous solution evaporated to dryness on a water bath. The residue obtained was dissolved in alcohol and precipitated with alcoholic chlorplatinic acid. A platinum analysis of the resulting salt indicated, with practically theoretical accuracy, the platinum salt of α -methylcholine; suggesting that either the acylation had not proceeded at all or complete saponification of the ester had resulted from the treatment with water.

The remainder of the original oily residue was taken up in cold water, extracted with ether, and a portion of the aqueous solution treated at once with aqueous chlorplatinic acid. A yellow, more or less waxy, precipitate separated which proved to be the platinum salt of the phenylacetyl derivative,



alcohol but soluble even in cold water. The attempt to concentrate the mother liquor on a water bath, however, resulted in decomposition. On heating the salt in a capillary tube it shows the effect, slightly, as low as 219° , turns dark gradually above 229° and decomposes with effervescence at about 245.7° – 246.7° (corrected).

Analysis for platinum by ignition resulted as follows:

0.1021 gram salt gave 0.0225 gram Pt.

	Calculated for	Found:
	$\text{C}_{12}\text{H}_{14}\text{O}_4\text{N}_3\text{Cl}_4\text{Pt}$:	
Pt.....	22.14	22.04

As in the case of the benzoyl derivative the gold salt of the phenyl-

acetyl derivative, $\left(\text{N} \begin{array}{l} \text{---} \text{CH}_3 \\ \text{---} \text{CH} \cdot \text{CH}_2 \text{OCOCH}_2 \text{C}_6\text{H}_5 \\ | \\ \text{CH}_3 \end{array} \right) \text{AuCl}_4$, separates

as an oil and could not readily be obtained in a condition favorable for melting point determination and analysis. It is readily soluble in hot water and slightly soluble in 95 per cent alcohol but appears to undergo decomposition in both cases as shown not only by analysis but also by marked change in melting point. One sample of the salt, undoubtedly impure, melted slowly over a wide range from about 140° – 160° , but on recrystallizing from hot water (yielding well-formed crystals) it melted at about 192° – 194° , which approximates the melting point of the gold salt of α -methylcholine itself, 197° – 198.5° (corrected). Similar observations were made on the behavior of the platinum salt. It appears therefore that the phenylacetyl derivative of α -methylcholine is not a very stable compound either in the form of the chloride, the platinum salt, or the gold salt.

For the purpose of comparison, both physiologically and chemically, with the choline derivative just described the corresponding derivative of " β -homocholine"⁴ (β -methylcholine) chloride was prepared:

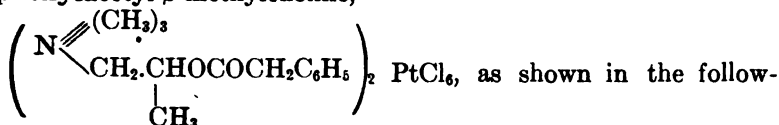
Phenylacetyl- β -methylcholine chloride (phenylacetyl- β -oxypropyltrimethylammonium chloride), $\text{Cl} \cdot \text{N} \begin{array}{l} \text{---} \text{CH}_3 \\ \text{---} \text{CH}_2 \text{CHOCOC}_6\text{H}_5 \\ | \\ \text{CH}_3 \end{array}$

This compound was prepared by heating a mixture of the corresponding choline chloride and phenylacetyl chloride (in excess) in a sealed tube to 100° (water bath) for about three hours. As with the other preparations described, treatment with dry ether precipitated an oily semi-solid product. A portion of this was converted into the platinum salt, analysis of which indicated a mixture of the unaltered β -methylcholine and the desired derivative.

An attempt to separate this mixture into its component salts by

⁴ Other acyl derivatives of " β -homocholine" have been prepared in this Laboratory and are briefly described by Hunt and Taveau in Hygienic Laboratory Bull. No. 73, U. S. Public Health Service.

fractional crystallization from hot water was successful. Two distinct fractions were obtained. The less soluble fraction showed a melting point of about 250°, corresponding to that reported by Hunt and Taveau⁵ for "β-homocholine" platinum salt. The more soluble (smaller) fraction melted at 216°–217° (corrected), and analysis for platinum indicated it to be the platinum salt of phenylacetyl-β-methylcholine,



ing data:

0.1052 gram salt gave 0.0233 gram Pt.

	Calculated for $\text{C}_{21}\text{H}_{40}\text{O}_4\text{N}_2\text{Cl}_6\text{Pt}$:	Found:
Pt.....	22.14	22.15

This experiment demonstrated in a striking way the greater stability of this particular derivative of β-methylcholine as compared with the same derivative of α-methylcholine (described above) under similar conditions, and suggests the possibility of a general rule to the effect that esters of secondary alcohols are less easily saponified than the same ester of isomeric primary alcohols.

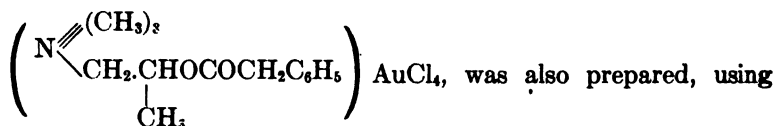
A second preparation of phenylacetyl-β-methylcholine, involving the same quantities of reagents as in the first, was made in a successful attempt to obtain a more quantitative reaction. The mixture in a sealed tube was heated in a boiling water bath for about seven hours. It was then transferred to a tube-furnace and heated to 150° for about one hour. The reaction product, treated as in previous preparations, was converted into the platinum salt, which without recrystallization analyzed as follows:

0.1076 gram salt gave 0.0238 gram Pt.

	Calculated for $\text{C}_{21}\text{H}_{40}\text{O}_4\text{N}_2\text{Cl}_6\text{Pt}$:	Found:
Pt.....	22.14	22.03

The gold salt of this derivative,

⁵ Hygienic Laboratory Bull. No. 73, U. S. Public Health Service, footnote, p. 33.



chloride of the base that had been standing in a vacuum desiccator for about four weeks. On adding chlorauric acid to an aqueous solution of the choline chloride the gold salt separated as a pale yellow oil which quickly became crystalline on rubbing. On heating in a capillary tube it sintered slowly above 60° , fused without flowing at 74.5° – 76° , became limpid and fairly clear at about 85° (not corrected). Analysis for gold by ignition resulted as follows:

0.1080 gram salt gave 0.0374 gram Au.

	Calculated for $\text{C}_{14}\text{H}_{22}\text{O}_2\text{NCl}_4\text{Au}$:	Found:
Au	34.28	34.63

From the aqueous mother liquor on standing a considerable further separation of flaky crystals resulted. In a melting point determination this fraction showed the same general behavior as the crystallized oil, but at a higher temperature, fusion to a mushy mass occurring at 86.5° – 88.5° instead of at 74.5° – 76° . Analysis disclosed the considerably higher gold content of 36.79 per cent. The crystallized oil fraction, which was slightly soluble in cold water and in cold absolute alcohol but readily soluble in these solvents hot, was dissolved in a little hot water from which on cooling it separated in flaky crystals. This fraction showed a further difference in melting point, fusing at 114.5° – 115.5° and becoming a clear limpid liquid at 118° (uncorrected). The remaining material was insufficient for analysis.

In view of the data derived from the platinum salt of this derivative the above described behavior of the gold salt would suggest decomposition of the gold salt rather than serious impurity in the original product, though there is the alternative possibility (which was not verified) of partial decomposition of the chloride of the base on long standing in a vacuum desiccator.

For comparison with the phenylacetyl derivatives already described the corresponding derivative of " γ -homocholine"

* Other acyl derivatives of " γ -homocholine" have been prepared in this laboratory and are briefly described by Hunt and Taveau in Hygienic Laboratory Bull. No. 73, U. S. Public Health Service.

$\text{Cl.N} \begin{array}{c} \diagup (\text{CH}_3)_3 \\ \diagdown \end{array} \text{CH}_2.\text{CH}_2.\text{CH}_2.\text{OCOCH}_2.\text{C}_6\text{H}_5$, was also prepared. This compound was prepared by the same method applied to the β -compound. Only two hours' heating in the water bath, however, appeared sufficient to induce complete reaction. As in other cases the crude reaction product was oily but on concentrating, in a vacuum desiccator, a portion of the aqueous solution, after extracting with ether, it gradually separated as a white flaky solid. Another portion of the aqueous solution was precipitated with chlorplatinic acid forming the platinum salt,

$\left(\text{N} \begin{array}{c} \diagup (\text{CH}_3)_3 \\ \diagdown \end{array} \text{CH}_2.\text{CH}_2.\text{CH}_2.\text{OCOCH}_2.\text{C}_6\text{H}_5 \right)_2 \text{PtCl}_6$. This salt is practically insoluble in alcohol and difficultly soluble in hot water from which it crystallizes in individual, and clusters of, stocky prisms. It melts sharply with decomposition at about 193° to 194° (corrected). On ignition:

0.1031 gram salt gave 0.0230 gram Pt.

	Calculated for $\text{C}_{23}\text{H}_{44}\text{O}_4\text{N}_2\text{Cl}_6\text{Pt}$:	Found:
Pt.....	22.14	22.31

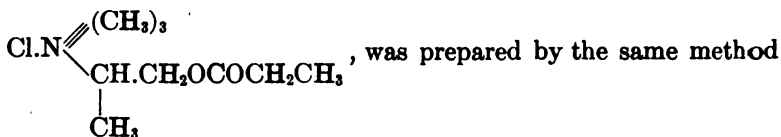
The gold salt of this derivative,

$\left(\text{N} \begin{array}{c} \diagup (\text{CH}_3)_3 \\ \diagdown \end{array} \text{CH}_2.\text{CH}_2.\text{CH}_2.\text{OCOCH}_2.\text{C}_6\text{H}_5 \right) \text{AuCl}_4$, was precipitated from aqueous solution with aqueous chlorauric acid and separated as a pale yellow, crystalline solid. It is nearly insoluble in cold water and rather difficultly soluble in warm water from which on cooling it separates in flaky crystals. The solubility in absolute alcohol is considerably greater than in water. It melts slowly with slight effervescence at 129° – 131° (corrected). On ignition:

0.1073 gram salt gave 0.0370 gram Au.

	Calculated for $\text{C}_{23}\text{H}_{44}\text{O}_4\text{NCl}_4\text{Au}$:	Found:
Au.....	34.28	34.48

The propionyl derivative of α -methylcholine chloride (propionyl- β -methylethoxytrimethylammonium chloride),



and procedure already fully described. Analysis of the crude gold and platinum salts indicated that the reaction was still not quite complete after heating the mixture of α -methylcholine and propionyl chloride (in excess) in a sealed tube for between three and four hours in a boiling water bath. No attempt was made to purify the gold salt, analysis of which, however, indicated a sufficient degree of purity for preliminary pharmacological purposes as shown in the following data:

0.2 gram salt gave 0.0776 gram Au.

	Calculated for $\text{C}_8\text{H}_{19}\text{O}_2\text{NCl}_2\text{Au}$:	Found:
Au.....	38.43	38.8

The platinum salt was purified by recrystallizing from hot water. The pure salt decomposes, under the conditions previously outlined (footnote 3), at about 231° – 232° (corrected). On ignition for platinum analysis:

0.1101 gram salt gave 0.0285 gram Pt.

	Calculated for $\text{C}_{11}\text{H}_{25}\text{O}_4\text{N}_2\text{Cl}_2\text{Pt}$:	Found:
Pt.....	25.77	25.88

Valeryl, monobromisocapryl, and palmityl derivatives of α -methylcholine were also prepared by the method described.

The valeryl derivative (valeryl- β -methylethoxytrimethylammonium chloride, $\text{Cl.N} \begin{array}{l} \text{//} (\text{CH}_3)_3 \\ \text{---} \text{CH} \cdot \text{CH}_2 \text{OCOCH} \\ | \quad \quad \quad \text{CH}_3 \\ \text{CH}_3 \quad \quad \quad \text{C}_2\text{H}_5 \end{array}$ was obtained in pure

form without difficulty. The platinum salt, precipitated from aqueous solution, on heating in a capillary tube gradually sinters above 219° and decomposes with effervescence at about 228° – 229° (corrected). Ignited for platinum:

0.1528 gram salt gave 0.0367 gram Pt.

	Calculated for $\text{C}_{22}\text{H}_{45}\text{O}_4\text{N}_2\text{Cl}_2\text{Pt}$:	Found:
Pt.....	23.99	24.05

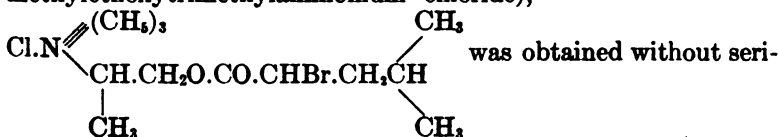
The gold salt separated as an oil from both aqueous and alcoholic solution. On standing over night at room temperature it crystallized (prisms). Heated in a capillary tube it sinters sharply at 72° and is completely melted on slowly heating to 75°. It is practically insoluble in cold water, slightly soluble in cold absolute alcohol and in hot water, and readily soluble in hot alcohol. Analysis for gold resulted as follows:

0.1224 gram salt gave 0.0449 gram Au.

	Calculated for $C_{11}H_{15}O_2NCl_2Au$:	Found:
Au.....	36.44	36.68

Subsequent investigation of the valeryl derivative, by means of platinum and gold preparations, indicated that it had deteriorated on long standing in a vacuum desiccator.

The monobromisocapronyl derivative (α -bromisocapronyl- β -methylethoxytrimethylammonium chloride),



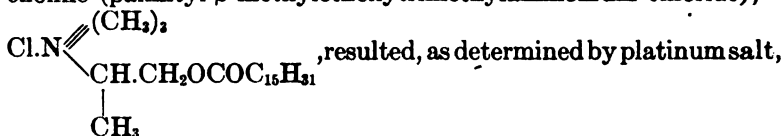
ous difficulty, though perhaps not perfectly pure, after about five hours' heating of the reacting mixture in the water bath. The platinum salt, freshly precipitated from aqueous solution, appeared of waxy consistency but became more granular on rubbing with a glass rod. Precipitated from alcoholic solution it was still more waxy in character. It is practically insoluble in absolute alcohol, cold or hot, and in cold water, but dissolves slowly in hot water from which on cooling it separates in a dense flocculent mass, isolated patches of which appeared, under the microscope, to be clusters of very fine needles. The clean dry salt, heated in a capillary tube, decomposes at about 226°–227° (corrected). On ignition for platinum:

0.1507 gram salt gave 0.0291 gram Pt.

	Calculated for $C_{20}H_{35}O_4N_2Cl_2Br_2Pt$:	Found:
Pt.....	19.52	19.31

The gold salt of this derivative, which separates from aqueous solution (and persists) as a reddish oil, could not be obtained in sufficient purity to give a satisfactory analysis.

A first attempt to prepare the palmityl derivative of α -methylcholine (palmityl- β -methylethoxytrimethylammonium chloride),

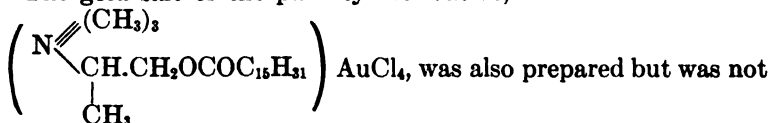


in absolute failure. The experiment was repeated taking special precautions to use dry reagents. The mixture, including a larger excess of palmityl chloride than in the first attempt, was heated in a water bath for about six hours and allowed to remain sealed and standing in the cooling bath over night. On pouring the reaction product into dry ether a finely divided white waxy solid, more or less oily, separated. This proved to be so much less soluble in water than the other acyl derivatives that preparation of the platinum salt from aqueous solution was impractical. Analysis of the platinum salt, precipitated from alcoholic solution, indicated the reaction product to be a mixture of unaltered α -methylcholine chloride and the palmityl derivative. The salt was treated with hot water and heated with stirring on a water bath. A comparatively small part of it dissolved readily, the rest remaining apparently unaffected even on the addition of more water. The dissolved portion, largely recovered by concentration of the mother liquor, gave analytical results for platinum which were practically theoretical for the platinum salt of the unaltered base. The undissolved portion proved to be, as indicated by analyses for platinum, the platinum salt of the palmityl derivative. It is practically insoluble in alcohol and in water, cold or hot. Heated in a capillary tube it decomposes with effervescence at about 240° – 241° . Duplicate analyses for platinum by ignition resulted as follows:

- I. 0.1005 gram salt gave 0.0172 gram Pt.
- II. 0.1006 gram salt gave 0.0173 gram Pt.

	Calculated for $\text{C}_{16}\text{H}_{35}\text{O}_4\text{N}_3\text{Cl}_2\text{Pt}$:	Found:	
		I.	II.
Pt.....	17.39	17.12	17.2

The gold salt of the palmityl derivative,

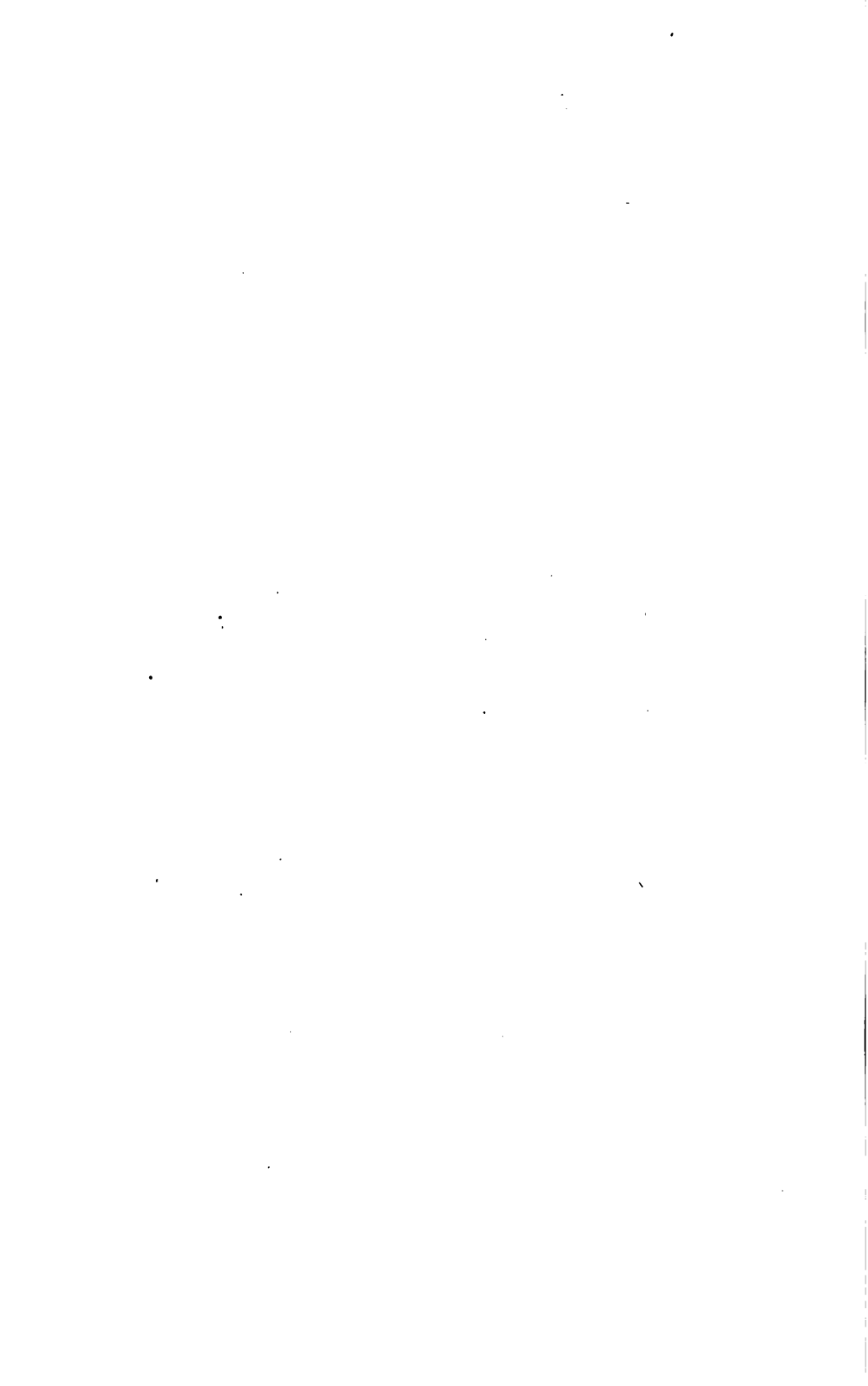


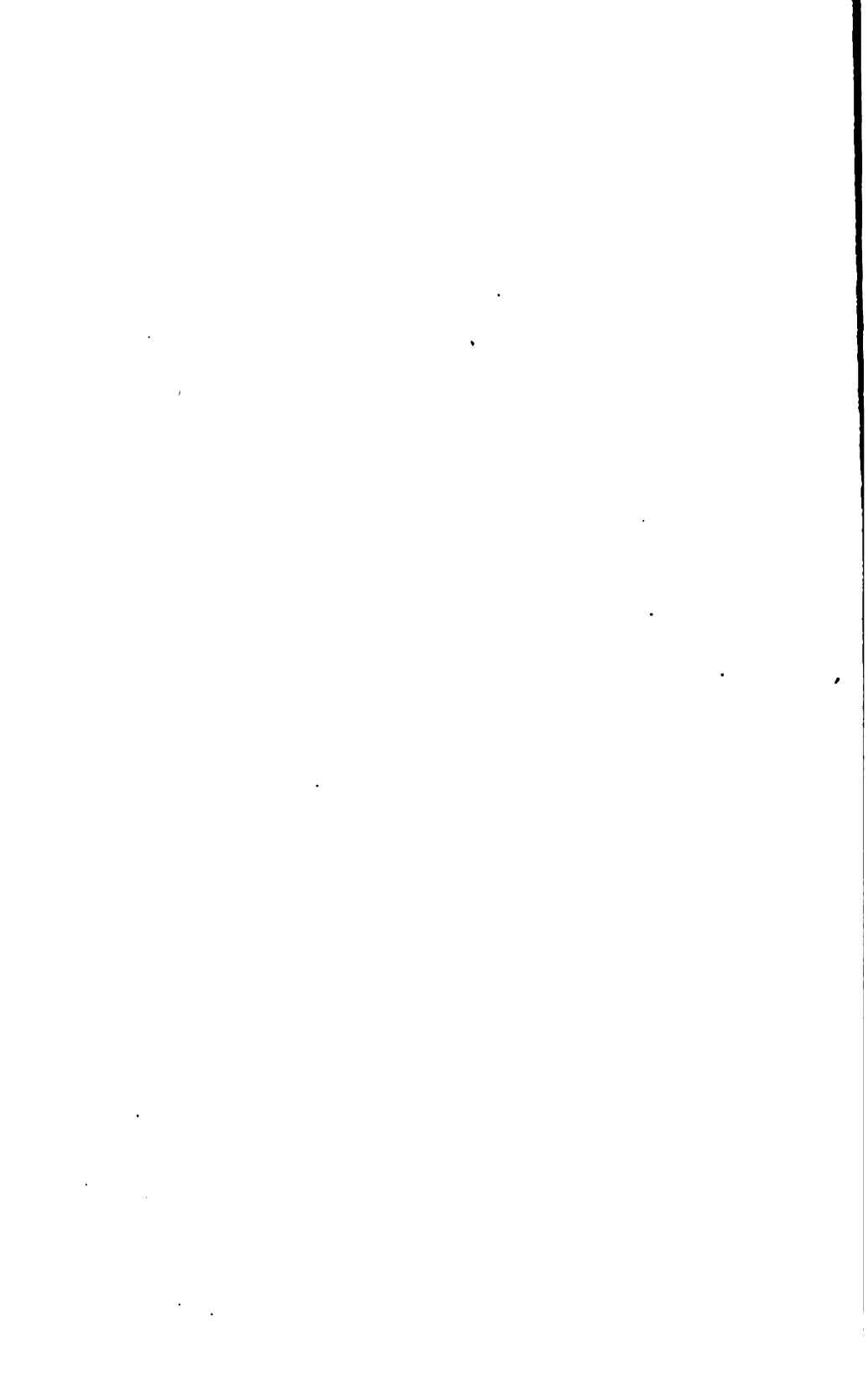
easily obtained pure. It is too soluble in absolute alcohol to be prepared in that medium or to be recrystallized from it, but when water is added to the alcoholic solution the salt separates as a pale yellow greasy precipitate, which was made more definitely crystalline by washing with ether. It is readily soluble in absolute alcohol, slightly soluble in water and in ether. It melts (not sharply) at about the same temperature as the gold salt of the valeryl derivative (72° – 75°). A large part of available salt was lost by decomposition in attempting to dry it in an air bath at about 80° . The small remainder was more carefully dried, and analyzed for gold by ignition (to constant weight):

0.0980 gram salt gave 0.0275 gram Au.

	Calculated for	
	$C_{22}H_{40}O_5NClAu$:	Found:
Au.....	28.37	28.06

Since the publication of the preliminary paper cited above the development of new compounds of the choline type has been continued in this laboratory, as opportunity permitted, with more or less success. Some of the results obtained in this later work will be submitted for publication at an early date.





A STUDY OF THE TRYPTIC PROTEOLYSIS OF CYNOSCION REGALIS.

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(Received for publication, September 3, 1912.)

It is frequently desired to compare the rate and course of digestion of various proteins by enzymes, according to methods which while not complicated will give distinctive and reliable results. Van Slyke's¹ method for the determination of amino-acids has been successfully applied by White and Crozier² to a comparison of the tryptic proteolysis of beef and several fish meats. The ease of manipulation of the apparatus, the brief time required for a determination and the regularity of the experimental data, makes the process generally useful. Also the conclusions drawn conform with those from metabolism experiments, making the results of still greater value.

The method which Sørensen³ has proposed for the estimation of amino-acids and polypeptides, by titration with caustic soda after the addition of formaldehyde, by its simplicity suggests itself for artificial digestion processes. It is the object of this article to show its application to the hydrolysis of *Cynoscion regalis* (squeteague, weakfish) by trypsin, and to compare the results with those obtained by Van Slyke's method.

The squeteague was boiled in water for one-quarter of an hour, allowed to drain from excess of liquid and preserved ice-cold. An analysis of two samples gave an average of 4.52 per cent N.

The digestion was carried on in 250 cc. volumetric flasks placed in a thermostat kept at a temperature of 37.5°C. Enough meat to furnish 1.5 grams of nitrogen was weighed out, ground up with water together with

¹ This *Journal*, ix, p. 185, 1911.

² *Journ. Amer. Chem. Soc.*, xxxiii, p. 2042, 1911.

³ *Biochem. Zeitschr.*, vii, p. 45, 1907.

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1 gram of trypsin, and this mixture poured into the flask. Twenty-five cubic centimeters of $\frac{N}{10}$ sodium hydroxide solution were added and the whole made up to 250 cc. with water. A little chloroform was then added. Trypsin is presumably most active in a medium which is alkaline with sodium carbonate, but the presence of this salt would interfere with the titration for the amino-acids where phenolphthalein must be used as an indicator, so that the alkalinity was ensured by the addition of the hydrate. Separate mixtures were made and analyses run in duplicate for the time periods of one, one-half, two, five and eight hours respectively. A sample, similar to the above but containing no trypsin, was also prepared and analyzed with the others according to the following method.

At the end of the desired time of digestion, the mixtures were filtered and aliquot portions of the filtrate taken for the various tests. Ten cubic centimeters were used for the determination of total soluble nitrogen by the Kjeldahl method, 10 cc. for the amount of amino nitrogen by Van Slyke's method and 20 cc. were treated with 10 cc. of 40 per cent formaldehyde solution and titrated to a distinct pink color with $\frac{N}{10}$ sodium hydroxide solution. We found that in every case an excellent end point was obtained, duplicate analyses agreeing within 0.2 per cent. Finally, 10 cc. of the filtrate from the digested fish were completely hydrolyzed by prolonged digestion on the water bath with 40 cc. of concentrated hydrochloric acid. This solution was evaporated to dryness, made up to 50 cc. with water, 10 cc. tested for amino nitrogen by Van Slyke's method and 20 cc. analyzed for amino-acids by Sørensen's method.

All the results obtained as above were corrected for amino nitrogen before and after complete hydrolysis, for total soluble nitrogen and for amino-acids as determined by titration with caustic soda, by carrying through the same experiments with the trypsin and alkali but with no protein. Correction was also made for the alkali required to neutralize the formaldehyde solution.

TABLE I.

*Total and amino nitrogen in solutions of Cynoscion regalis.
Hydrolyzed by trypsin.*

TIME IN HOURS	SOLUBLE N	INSOLUBLE N	AMINO N	AMINO N AFTER HYDROLYSIS BY HCl	AVERAGE SIZE OF PEPTIDES	100 × SOLUBLE N TOTAL N	100 × AMINO N SOLUBLE N
0	0.170	1.330	0.017	0.056	3.29	11.32	10.00
$\frac{1}{2}$	1.115	0.385	0.230	0.464	2.02	74.32	20.63
1	1.175	0.340	0.250	0.464	1.86	78.34	21.27
2	1.173	0.327	0.289	0.464	1.61	78.20	24.63
5	1.361	0.139	0.357			90.72	26.23
8	1.432	0.068	0.406	0.646	1.59	95.45	28.35

In table I are presented the results obtained by applying Van Slyke's method to the tryptic proteolysis of squeteague. The average size of the peptides was calculated by dividing the amount of amino nitrogen present after complete hydrolysis with hydrochloric acid by that in the solution before such hydrolysis. The last two columns of data show the increase with time of the proportion of soluble to insoluble nitrogen and of amino to total soluble nitrogen respectively.

The average results of duplicate analyses are given.

TABLE II.

*Analysis by Sørensen's method of solutions of Cynoscion regalis.
Hydrolyzed by trypsin.*

TIME IN HOURS	CC. $\frac{N}{10}$ NaOH REQUIRED	CC. $\frac{N}{10}$ NaOH REQUIRED AFTER HYDROLYSIS WITH HCl	AVERAGE SIZE OF PEPTIDES	AMINO N CALCU- LATED	AMINO N CALCULATED $100 \times \frac{\text{AMINO N}}{\text{SOLUBLE N}}$
0	24.85	125.0	5.02	0.035	20.52
$\frac{1}{2}$	182.8	536.8	2.94	0.257	23.02
1	213.8	598.0	2.80	0.300	25.87
2	232.9			0.327	27.87
5	293.5	776.0	2.64	0.412	30.28
8	314.8	787.1	2.50	0.442	30.87

In table II are given the average results of the analysis of the proteolyzed solutions according to Sørensen's method. In column 2 the figures represent cubic centimeters of $\frac{N}{10}$ sodium hydroxide solution required for neutralization after addition of formaldehyde solution. Column 3 is the same for the solutions after complete hydrolysis with hydrochloric acid. The next column gives the ratio of the latter to the former. In column 5 are given figures for amino nitrogen calculated from the data in column 2, while in the last column the values of the calculated amino nitrogen are expressed as percentages of the total soluble nitrogen.

From table I it is seen from the ratio of the soluble to total nitrogen that the fish meat goes very rapidly into solution, 74.32 per cent of the nitrogen being in solution at the end of one-half hour's digestion. Solution, however, is not complete in eight hours'

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time, a fact which is *apparently* not in harmony with the results of White and Crozier,⁴ who found with the proteins they studied that all the nitrogen was in the soluble form in from four to eight hours. The trypsin used, a commercial sample, was of the same activity in both series of experiments. These latter experiments were carried on in a medium made alkaline with sodium carbonate, while the experiments described in this article required sodium hydroxide for reasons already stated. Schierbeck⁵ has proved that the action of trypsin in digesting proteins is accelerated by the presence of carbon dioxide in solutions which are slightly alkaline,

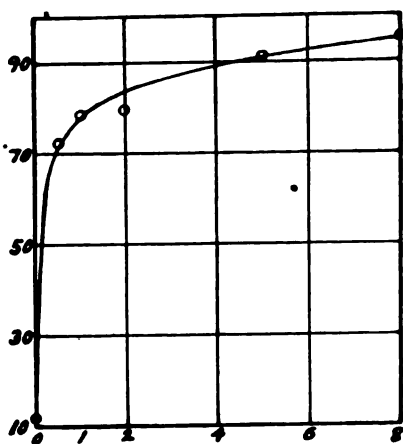


FIG. 1. Abscissae—Time in hours.
Ordinates— $100 \times \frac{\text{Soluble Nitrogen}}{\text{Total Nitrogen}}$.

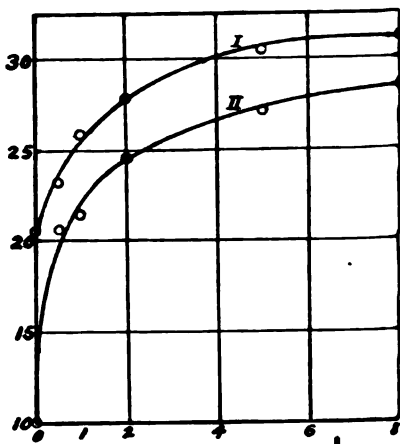


FIG. 2. Abscissae—Time in hours.
Ordinates— $100 \times \frac{\text{Amino Nitrogen}}{\text{Soluble Nitrogen}}$.
I By Sørensen's method.
II By Van Slyke's method.

and it is very probably at least due partly to this fact that the above differences are found. The variation of the proportion of soluble to total nitrogen with increase of time is shown graphically in figure 1. Extrapolation of the curve would indicate that the squeteague would be completely dissolved in about fourteen hours.

White and Crozier have shown that their artificial digestion experiments gave results agreeing closely with those obtained from

⁴ *Loc. cit.*

⁵ *Skand. Arch. f. Physiol.*, iii. p. 344, 1892.

metabolism work with dogs, rates of digestion of different proteins being in the same relation to each other. Van Slyke and White,⁶ in a study of the relation between the digestion and retention of ingested proteins, found that squeteague is digested more slowly than either beef or cod. From the above facts, it is fair to conclude that the tardy solution of the squeteague by trypsin, shown by our data, is not alone due to the absence of carbon dioxide, but is a consequence of the inherent nature of the protein itself.

The amino nitrogen in solution increases of course with length of time. The average size of the peptides split off from the protein should be especially noted. From these data and the experiments of White and Crozier it is evident that the proteins studied break down into simple cleavage products practically *as soon as they go into solution*. At the end of one-half hour's digestion the average size of the peptides is only 2.02—the cleavage products on the whole are indicated to be amino-acids. The cleavage of certain proteins by trypsin has been intimately studied, and it is known that some amino-acids are readily formed while others are produced very slowly or not at all. The above results, however, show that the greater portion of the amino nitrogen in solution exists in bodies of exceedingly simple character. The significance of this, physiologically, cannot be pointed out here but will be reserved for future discussion.

The relation of the amino to the total soluble nitrogen is shown in figure 2. After eight hours' digestion only 28.35 per cent of the nitrogen is in the amino form. This is a confirmation of earlier work which, as just mentioned, has shown that there are certain substances which resist the hydrolytic action of trypsin altogether. The increase in the proportion of amino nitrogen during the eight hours' digestion is seen to be very slight, and we must conclude therefore that cleavage of the meat, while yielding amino bodies of a simple nature, leaves the greater part of the soluble nitrogen combined in substances which are extremely stable.

The same conclusions may be drawn from the results of our experiments involving Sørensen's method. In figure 2 the curves are of the same slope, although of course only approximate figures are expected on assuming the presence of one amino group for

⁶ *This Journal*, ix, p. 226, 1911.

each carboxyl group indicated by the sodium hydroxide required. The amino nitrogen thus estimated is regularly greater than that directly determined by the nitrous acid method, and the peptides as computed are about 1.5 times as large. It is possible that in such calculations, this effect is produced by the presence of such monamino-dicarboxylic compounds as glutamic acid. The discrepancy in the results is not of such a magnitude as to prevent deducing rigid conclusions concerning the rate and general course of digestion of such proteins as the one under investigation.

SUMMARY.

1. Sørensen's method for the determination of amino-acids was applied to a study of the tryptic digestion of *Cynoscion regalis*. The results were found to be regular and in accord with those obtained by the nitrous acid method of analysis for amino nitrogen.
2. The relatively low rate at which the protein is made soluble agrees with the results of metabolism experiments.
3. Very low cleavage products are formed as soon as the protein goes into solution, the average size of the peptides being 2.02 after one-half hour's digestion.
4. There is a very stable nitrogen complex which is not attacked by trypsin.

STUDIES ON THE FORMATION OF GLYCOCOLL IN THE BODY. II.

BY ALBERT A. EPSTEIN AND SAMUEL BOOKMAN.

(From the Laboratory of Physiological Chemistry, Pathological Department, Mount Sinai Hospital, New York City.)

(Received for publication, September 6, 1912.)

Benzoic acid, circulating in the fluids of the body, combines with glycocoll and removes this intermediary product of protein metabolism from further decomposition. The extent to which the elimination of glycocoll can thus occur, depends upon the glycocoll which is available, the amount of benzoic acid present and the rapidity with which the hippuric acid synthesis takes place. There is no reason to believe that a reserve of free or preformed glycocoll, sufficient to account for the quantities of hippuric acid eliminated in the urine, exists in the body. The apparent independence of the process of glycocoll formation from the general nitrogenous metabolism in the body under certain conditions (such as pure carbohydrate feeding), which we have demonstrated in our previous investigation,¹ points strongly to the conclusion that most of the glycocoll obtained as hippuric acid is the result of a synthetic production of the substance in the body.

The attempts made by a number of investigators to cause the synthesis of glycocoll in the body from simpler substances have thus far failed. On the other hand, in the absence of such evidence, the facts at our disposal also justify the belief that glycocoll can arise from the higher amino-acids. Magnus-Levy² regards leucine as being capable of conversion into glycocoll.

He concludes by computation on a theoretical basis that most of the glycocoll obtained in benzoic acid experiments can arise

¹ Epstein and Bookman: this *Journal*, x, p. 353, 1911.

² Magnus-Levy: *Münch. med. Wochenschr.*, lii, p. 2168, 1905.

from the leucine radical of protein. Free leucine however does not yield glycocoll.³ It is only when given as a benzoyl compound that it appears to be entirely converted into glycocoll. This deduction is based principally upon two facts, namely, that practically no benzoyl leucine appears, as such, in the urine after administration of large amounts of the substance and that other benzoylized amino-acids do not lead to the production of hippuric acid.⁴

If we accept these facts then we must at once conclude that leucine as such does not appear as an intermediary substance in the course of glycocoll production; and that the leucine-bearing portion in protein undergoes a different form of decomposition in the presence of benzoic acid than that which leads to the liberation of leucine itself. This gains support from the facts elicited in our previous study that in the course of the production of glycocoll, following the administration of benzoic acid, there does not occur any massive decomposition of protein comparable with that which accompanies phosphorus poisoning and the consequent liberation of leucine and other amino-acids.

In brief, if the leucine radical is capable of yielding glycocoll in response to benzoic acid, it must be by some process which does not affect the other protein radicals to any appreciable extent. Under the conditions of experimentation recorded by Magnus-Levy, it seems possible that the increased glycocoll output as hippuric acid,⁵ following the administration of benzoyl leucine, is not the result of a direct conversion of leucine into glycocoll. Benzoic acid does not couple leucine in the body. The leucine however is decomposed. The effect of a primary benzoylization *in vitro* upon leucine may therefore be such as to render it subject to complete decomposition in the body into substances which are ultimately synthesized into glycocoll. The final product (glycocoll) must then be regarded as the result not only of a cleavage process but of a synthesis as well. On the other hand, it is also conceivable that the leucine of the benzoyl compound undergoes complete decomposition without taking part in the production

³ Magnus-Levy: *loc. cit.*; Cohn: *Archiv f. exp. Path. u. Pharm.*, xlviii, p. 177, 1902.

⁴ Magnus-Levy: *loc. cit.*

⁵ Magnus-Levy: *loc. cit.*

of glycocoll; and the benzoyl radical which is set free is coupled with glycocoll furnished by the body in much the same manner as benzoic acid.

It was with the object of inquiring into this particular question that the present investigation was made. To determine this, three sets of experiments were performed. In the first of these, various amounts of benzoic acid were administered to rabbits; after the maximum output of hippuric acid was established, leucine was given subcutaneously and the effect upon the production of hippuric acid was studied.

Condensed Table 1. Daily average for each period.

PERIOD	BENZOIC ACID	LEUCINE N	URINE			Extra N
			Total N	Hippuric N	Hippuric N	
	grams	gram	gram	gram	per cent	gram
I	0	0	0.428	0.018	4+	
II	2.087	0.	0.705	0.135	19	0.142
III	2.087	0.100	0.784	0.118	15	0.238
IV	0	0	0.524	0.020	4—	0.076

The results obtained in these experiments are in accord with those of Magnus-Levy⁶ and of Cohn.⁷ Leucine evidently does not increase the glycocoll output. It appears that the amount of hippuric acid eliminated in the leucine period (III, table 1) is considerably less than that in the control period (II, table 1). The leucine nitrogen appears as extra nitrogen, but not as leucine: free leucine could not be detected in the urine.

In the second set of experiments, rabbits were given definite amounts of benzoic acid and the maximum hippuric acid output was established; then the dose of benzoic acid was increased and the effect of this increase upon the total hippuric acid output noted. Subsequently the animals were given the original quantity of benzoic acid and, in addition, benzoyl-leucine was given in such amounts that the benzoyl radical was the molecular equivalent of the extra dose of benzoic acid.

⁶ *Loc. cit.*

⁷ *Loc. cit.*

Condensed Table 2. Daily average for each period.

PERIOD	BENZOIC ACID	BENZOYL-LEUCINE		URINE			
				Total N	Hippuric N	Hippuric N	Extra N
	grams	gram	gram	gram	gram	per cent	gram
I	0			0.548	0.012	2.5	
II	2.5			0.816	0.108	13	0.160
III	3.125			0.922	0.198	19	0.176
IV	2.5			0.863	0.114	13	0.213
V	2.5	0.519+	0.688	0.868	0.234	27	0.084
VI	2.5			1.442	0.189	13	0.705

On comparing the benzoyl-leucine periods with their corresponding control periods in these experiments we find that a much greater output of hippuric nitrogen occurs in the benzoyl-leucine periods. This increment is much greater than the leucine radical of the benzoyl compound could possibly furnish.

In the first set (table 2) the hippuric nitrogen of the first benzoic period (II, in which the animal received 2.5 grams of benzoic acid daily) amounts to about 13 per cent of the total nitrogen. In the second benzoic period (III, when the dose is increased to 3.125 grams daily) the hippuric nitrogen eliminated comprises 19 per cent of the total nitrogen. In other words, the additional 0.625 gram of benzoic acid caused an increase in the hippuric nitrogen output amounting to 6 per cent of the total nitrogen. Upon returning to the original dose of benzoic acid (2.5 grams daily) in the third period, the hippuric nitrogen output is again 13 per cent of the total. In the following period (V, table 2), when benzoyl-leucine is given in place of the extra 0.625 gram of benzoic acid, the hippuric nitrogen rises to 27 per cent of the total. If we allow 19 per cent of this for the benzoic acid equivalent, then there is a balance of 8 per cent nitrogen to the credit of the leucine. This is almost the exact equivalent of nitrogen furnished by the leucine (0.094 gram).

Although the results of the second set of benzoyl-leucine experiments differ from the first in a number of details, they point nevertheless to the same conclusion. In the first benzoic period (II, table 3) the hippuric nitrogen constitutes 18 per cent of the total eliminated in the urine. An additional 0.5 gram of benzoic acid

daily does not seem to cause any greater output of hippuric nitrogen—which in the third period (III, table 3) comprises 16 per cent of the total nitrogen. Upon returning to the original dose of benzoic acid (IV, table 3) the hippuric nitrogen remains at the same level, 16 per cent of the total. But when, in addition to the benzoic acid, the benzoyl-leucine is given in such amounts that the benzoyl radical is equivalent to 0.5 gram of benzoic acid, the hippuric nitrogen rises and comprises 37 per cent of the total urinary nitrogen. In other words, an increase of 21 per cent is noted in this period (V, table 3) over the corresponding control period (III, table 3). Of this amount the leucine could furnish nearly 11 per cent or half of the extra hippuric nitrogen.

Condensed Table 3. Daily average for each period.

PERIOD	BENZOIC ACID	BENZOYL-LEUCINE		URINE			
				Total N	Hippuric N	Hippuric N	Extra N
	grams	gram	gram	gram	gram	per cent	gram
I	0			0.565	0.017	3	
II	2.0			0.588	0.104	18	
III	2.5			0.604	0.096	16	
IV	2.0			0.625	0.099	16	
V	2.0	0.415+	0.550	0.640	0.240	37	
VI	2.0			0.738	0.125	17	0.048
VII	0			0.544	0.016	3	

On the basis of the preceding experiment (table 2) the other half of the extra hippuric nitrogen eliminated is probably due to the independent action of the benzoyl radical. These results therefore indicate that benzoyl-leucine possesses a double efficiency in the production of hippuric acid: first by the conversion of leucine into glycocoll and secondly by the independent action of the benzoyl radical in coupling up available glycocoll.

In view of the fact that free leucine does not yield glycocoll, whereas benzoylized leucine evidently does, it seems probable that the leucine does not occur as an intermediary body in the normal metabolism. In the third series of experiments an attempt is made to ascertain whether, under conditions in which leucine occurs in the native state as an intermediary product of metabolism, the formation of glycocoll is influenced in any way.

Formation of Glycocoll in the Body

With a view to this end, a study of the production of hippuric acid in animals poisoned with phosphorus was made.

Condensed Table 4. Daily average for each period. Control.

PERIOD	BENZOIC ACID	PHOS- PHORUS OIL, 1 PER CENT	URINE			
			Total N	Hippuric N	Ammonia N	Extra N
	gram	cc.	gram	gram	gram	gram
I	0	0	0.709	0.012	0.030	
II	0	0.9	0.837	0.016	0.029	0.127

Without the administration of benzoic acid, poisoning with phosphorus does not increase the hippuric acid output. Traces of leucine however are found in the urine.

Condensed Table 5. Daily average for each period.

PERIOD	BENZOIC ACID	PHOS- PHORUS OIL, 1 PER CENT	URINE			
			Total N	Hippuric N	Ammonia N	Extra N
	grams	cc.	gram	gram	gram	gram
I	0	0	0.509	0.022	0	
II	2.087	0	0.754	0.092	0.027	0.245
III	2.087	0.6	0.882	0.092	0.030	0.373
IV	2.087	1.2	1.292	0.096	0.030	0.783

Condensed Table 6. Daily average for each period.

PERIOD	BENZOIC ACID	PHOS- PHORUS OIL, 1 PER CENT	URINE			
			Total N	Hippuric N	Ammonia N	Extra N
	grams	cc.	gram	gram	gram	gram
I	0	0	0.815	0.035	0.032	
II	2.5	0	0.999	0.151	0.030	0.184
III	2.5	0.6	1.038	0.113	0.036	0.223

Phosphorus poisoning in a feeding animal, receiving benzoic acid, causes no change in the amount of hippuric acid (and hence glycocoll) eliminated, although it causes a very striking increase in the total nitrogenous metabolism. Leucine could not be detected in the urine. This result is constant, as repeated experi-

ments show (tables 5 and 6). The hippuric acid output in the poisoned animal can fall below that of the control period, whereas the total urinary nitrogen may rise very high (table 6).

The result is somewhat different when a similar experiment is performed on a fasting animal.

Condensed Table 7. Daily average for each period.

PERIOD	STATUS	BENZOIC ACID	PHOS- PHORUS OIL, 1 PER CENT	URINE		
				Total N	Hippuric N	Extra N
		grams	cc.	grams	gram	gram
I	Feeding	0	0	0.681	0.013	
II	Feeding	2.0	0	0.984	0.083	0.233
III	Fasting	2.0	0	1.103	0.072	0.462
IV	Feeding	2.0	0	0.880	0.118	0.094
V	Fasting	2.0	0.9	1.422	0.123	0.631

The influence of the three factors, namely, fasting, benzoic acid and phosphorus (singly and collectively), upon the course of protein metabolism and glycocholate formation, is clearly illustrated in this experiment (table 7). In the fasting period the production of hippuric acid caused by the benzoic acid is less than in the normal control period, although the total nitrogen output is high. Upon returning to normal feeding the total nitrogen drops below and the hippuric nitrogen rises considerably above the preceding period. With the development of phosphorus poisoning (in a fasting animal, receiving benzoic acid) and its attendant massive protein decomposition, there occurs an even greater output of urinary nitrogen than in any of the preceding periods; and the hippuric nitrogen also rises above the control fasting period. The urine shows the presence of traces of leucine.

The increased elimination of hippuric acid, observed in the experiment just described, must be attributed to the excessive breakdown of protein, additional glycocholate probably being derived from that source. The very large amount of extra nitrogen eliminated is an evidence of this. The absence of any very appreciable amount of leucine may be interpreted in one of two ways: viz., that the leucine leads to the production of glycocholate on the

one hand, or, that it leads to the formation of nitrogenous bodies other than glycocoll. From the evidence furnished by these experiments it cannot be said, however, that native leucine plays a direct rôle in the production of glycocoll in the body.

SUMMARY.

Free leucine does not yield glycocoll although it undergoes decomposition in the body.

When benzoyl-leucine is given, in addition to a definite dose of benzoic acid, the output of hippuric acid is much greater than the leucine, alone, allows. It seems that the increase in the total glycocoll eliminated is partly due to the leucine and partly to the independent action of the benzoyl radical.

Phosphorus poisoning in a normal animal causes no increased production of glycocoll or hippuric acid. Phosphorus poisoning in an animal receiving benzoic acid also fails to increase the hippuric acid output. The fasting animal, when treated with benzoic acid and then poisoned with phosphorus, eliminates more glycocoll than the non-fasting animal, but the increase must be attributed to a greater amount of the substance being available for synthesis with benzoic acid as a result of a massive decomposition of protein.

In conclusion it may be said that the experiments here recorded do not afford any definite evidence of the conversion of native leucine into glycocoll. In the absence of such evidence it appears likely that much of the glycocoll liberated upon feeding benzoic acid is the result of a synthetic process in the body.

The detailed results of the foregoing experiments are given in the accompanying tables.

DATE	PERIOD	WEIGHT grams	FOOD (CARROTS) grams	DIBASIC ACID grams	LEUCINE N gram	TOTAL N gram	TOTAL N DAILY AVERAGE gram	NIPPURIC N	
								gram	gram
Oct. 28 29 30 31	I	2000	300			0.460	0.428		0.018
		1950	300			0.427			
		1970	300			0.399			
		1970	300			0.406			
Nov. 1 2 3 4 5 6 7 8 9 10	II	1950	300	2.087		0.455	0.705	0.177	0.135
		1900	300	2.087		0.791		0.128	
		1950	300	2.087		0.651		0.152	
		1930	300	2.087		0.616		0.105	
		2000	300	2.087		0.696		0.111	
		1990	300	2.087		0.819		0.170	
		1990	300	2.087		0.707		0.130	
		1990	300	2.087		0.994		0.140	
		2000	300	2.087		0.665		0.114	
		1930	300	2.087		0.651		0.124	
11 12 13	III	2000	300	2.087	0.087	0.959	0.784	0.110	0.118
		2000	300	2.087	0.106	0.735		0.136	
		1930	300	2.087	0.106	0.658		0.108	
14 15 16 17 18 19	IV	1870	300	2.087		0.735	0.711	0.046	0.088
		1850	300	2.087		0.875		0.058	
		1820	300	2.087		0.721		0.094	
		1870	300	2.087		0.609		0.112	
		1850	300	2.087		0.644		0.114	
		1870	300	2.087		0.686		0.105	
20 21 22	V	1780	300			0.547	0.524	0.031	0.020
		1780	300			0.528		0.015	
		1790	300			0.508		0.014	

TABLE 2.
Rabbit 7. Benzoyl-leucine experiment.

DATE	PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	BENZOYL	LEUCINE	TOTAL N	HIPPURIC N DAILY AVERAGE	TOTAL N DAILY AVERAGE
Feb. 24	I		grams	grams	gram	grams	gram	gram	gram
25			300			0.567			
26			300	0		0.532		0.012	0.548
			300			0.546			
27	II		300	2.5		0.749			
28			300	2.5		0.707		0.085	0.730
29			300	2.5		0.735			
March 1	III		300	2.5		1.575			
2			300	2.5		0.791		0.132	0.902
3			300	2.5		0.861			
4			300	2.5		0.480			
5	IV		300	3.125		0.476			
6			300	3.125		1.260		0.198	0.922
7			300	3.125		1.029			
8	V		300	2.5		0.665			
9			300	2.5		0.952			
10			300	2.5		0.889			
11			300	2.5		0.553			
12			300	2.5		1.113		0.057	0.863
13			300	2.5		0.574			
14			300	2.5		1.113			
15			300	2.5		1.050			
16	VI		300	*2.5+	0.519	0.888	0.868	0.234	0.868
17	VII		300	2.5		1.442	1.442	0.189	1.442

Records lost.

Rabbit 10. Benzoyl-leucine experiment.

DATE	PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	BENZOTL	LEUCINE	TOTAL N	TOTAL N DAILY AVERAGE	HIPPURIC N DAILY AVERAGE
March 21			grams	grams	gram	gram	grams	gram	gram
22			300				1.353		
			300				0.399		
23	I		300	0			0.539		0.017
24			300	0			0.539	0.565	
25			300	0			0.616		
26	II		300	2.000			0.420		
27			300	2.000			0.672	0.588	0.104
28			300	2.000			0.567		
29			300	2.000			0.693		
30	III		300	2.500			0.525		
30			300	2.500			0.609	0.604	0.096
1			300	2.500			0.679		
2	IV		300	2.000			0.686		
3			300	2.000			0.448	0.625	0.099
4			300	2.000			0.742		
5	V		300	*2.000+	0.415	0.550	0.672	0.640	0.240
6			300	2.000	0.415	0.550	0.609		
7	VI		300	2.000			0.756		
8			300	2.000			0.651	0.738	0.125
9			300	2.000			0.798		
10	VII		300	0			0.616		
11			300	0			0.456	0.544	0.016
12			300	0			0.560		

Records lost.

*Equivalent to 2.500 grams benzoic acid.

TABLE 4.
Rabbit 2. Phosphorus poisoning control experiment.

DATE	PERIOD	WEIGHT grams	FOOD (CARROTS) grams	BENZOIC ACID grams	PHOSPHORUS OIL, 1 PER CENT cc.	TOTAL N gram	AMMONIA N gram	TOTAL N DAILY AVERAGE gram	MISSING N DAILY AVERAGE gram
Dec. 8			300			0.674	0.021		
9	I					0.672	0.034	0.709	0.012
10						0.728	0.00		
11		1910				0.727	0.028		
12	II	1970			0.6	0.630	0.029		
13		1930			0.6	0.896	0.029	0.837	0.016
14		1870			1.2	0.914	0.028		
*15		1770	230		1.2	0.890	0.031		

*Traces of leucine found in urine.

TABLE 3
Rabbit 3. Phosphorus poisoning experiment.

DATE	PERIOD	WEIGHT grams	FOOD (CARROTS)	BENZOIC ACID	PHOS- PHORUS OIL, 1 PER CENT	TOTAL N	AMMONIA N	TOTAL N DAILY AVERAGE	HIPPURIC N DAILY AVERAGE	HIPPURIC N DAILY AVERAGE
Nov. 29	I	1830	300		cc.	grams	gram	grams	gram	gram
30		1810	300			0.462		0.509	0.022	
Dec. 1		1860	300			0.518				
				0.546						
2	II	1850	300	2.087		0.700				0.081
3		1850	300	2.087		0.672			0.081	
4		1810	300	2.087		0.756				
5		1860	300	2.087		0.784	0.028	0.754	0.078	
6		1800	300	2.087		0.784	0.028		0.089	
7		1830	300	2.087		0.728	0.034		0.088	
8		1820	300	2.087		0.560	0.025		0.093	0.092
9		1800	300	2.087		0.714	0.025		0.090	
10		1800	300	2.087		0.914	0.017		0.111	
11		1770	300	2.087		0.924	0.029		0.093	
12	III	1770	300	2.087	0.6	0.882	0.022		0.109	0.092
13		1780	300	2.087	0.6	0.882	0.029	0.882	0.086	
14	*IV	1730	300	2.087	1.2	1.358	0.029	1.292	0.126	0.096
15	*V	1700	160	2.087	1.2	1.226	0.041		0.067	

*Leucine found in the urine of phosphorus period.

TABLE 6.
Rabbit 4. Phosphorus poisoning experiment.

DATE	PERIOD	WEIGHT grams	FOOD grams	BENZOIC ACID grams	FROM- PHOSPHORUS OIL, 1 PER CENT cc.	TOTAL N grams	TOTAL N DAILY AVERAGE grams	HIPPURIC N DAILY AVERAGE grams
January 12	I	2650	300			0.861	0.815	0.035
13		2570	300			0.903		
14		2580	300			0.756		
15		2580	300			0.742		
16	II	2520	300	2.5		0.770	0.999	0.151
17		2510	300	2.5		1.274		
18		2460	200	2.5		1.253		
19		2440	240	2.5		0.700		
20	III	2470	200	2.5	0.6	1.253	1.038	0.113
21		2450	200	2.5	0.6	1.155		
22		2220	120	2.5	0.6	0.770		
23		2220	90	2.5	0.6	0.973		
24*								

* Animal died.

Rabbit 5. Phosphorus poisoning experiments.

DATE	PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	PHOSPHORUS OIL PER CENT	TOTAL N	TOTAL N DAILY AVERAGE	HIPPURIC N	DAILY EXTRA N
		grams	grams	grams	cc.	grams	grams	grams	grams
Dec. 28	I	2220	300						
29		2260	300						
30		2260	300			0.630			
31		2260	300			0.896	0.681	0.013	
Jan. 1	II	2180	300			0.584			
2		2200	300			0.616			
3		2200	300	2.000		1.036			0.230
4		2200	300	2.000		0.896		0.083	
5	III	2210	300	2.000		0.644	0.984		
6		2200	300	2.000		0.644			
7		2200	300	2.000		1.701			
8		2065	Fasting	2.000		1.025			0.463
9	IV	2045	Fasting	2.000		1.025	1.103	0.072	
10		2040	Fasting	2.000		1.025			
11		1940	Fasting	2.000		1.337			
12		1960	300	2.000		0.952			0.096
13	V	2100	300	2.000		0.868	0.880	0.118	
14		2110	300	2.000					
15		2100	300	2.000		0.882			
16		2100	300	2.000		0.819			
17		1950	Fasting	2.000	0.9	1.155			0.631
18		1880	Fasting	2.000	0.9	0.730	1.422	0.123	
18 to 19			Fasting	2.000	0.6	1.435			

56 hours.

COMPARATIVE EFFICIENCY FOR GROWTH OF THE TOTAL NITROGEN FROM ALFALFA HAY AND CORN GRAIN.¹

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It is well known that alfalfa hay, as well as other hays and root crops, contains a considerable part of its nitrogen in non-protein form. In alfalfa hay this may amount to 25 per cent of the total nitrogen, as determined by Stutzer's reagent or the tannic acid method. Just what the nature of this nitrogen is has not been determined, but it is usually assumed to be a mixture of acid-amides and amino-acids and is generally described under the term "amides." Attempts in this laboratory to isolate individual amino-acids from the water extract of alfalfa hay have failed, but further study of this extract, with the use of the Van Slyke nitrous acid method, shows the presence of free amino groups; but by no means is all of the "amide nitrogen" in the form of simple amides or amino-acids. A large proportion is still more complex than these and yet non-precipitable by such reagents as basic lead acetate, tannic acid, etc. (The results of a detailed chemical study in this laboratory of the water extract of alfalfa hay will be published shortly.)

There is a difference of opinion as to the real worth of "amide nitrogen" for either maintenance, growth or milk production. Armsby³ in his latest tables of feeding standards, excludes it entirely

¹ Published by permission of the Director of the Agricultural Experiment Station.

² Credit is due Mr. P. H. Wessels for part of the analytical work reported in this investigation.

³ Farmers Bulletin 346, U. S. Department of Agriculture.

from his calculations of the available nitrogen. On the other hand, Henry⁴ and Jordan,⁵ at least in their tables of the composition of feeds, give it full value. Nor has scientific inquiry as to its worth been wanting. The whole question is to some extent bound up with the question of the nature and extent of protein hydrolysis in animal digestion. It appears from the work of Cohnheim,⁶ Kutscher and Seemann⁷ and Abderhalden, Baumann and London⁸ that digestion proceeds to the formation of abiuret products in the form of polypeptides and the comparatively simple monoamino- and diamino-acids. Feeding experiments with pre-digested protein have also thrown light on this question. Loewy⁹ showed that protein, digested until the biuret reaction was absent, was capable not only of maintaining life, but of maintaining nitrogen equilibrium and even of leading to nitrogen retention. Henderson and Dean¹⁰ used the products of acid hydrolysis of proteins. They also record nitrogen retention. Others, as Henriques and Hanson,¹¹ were unable to get as good results with the products of acid hydrolysis as when the hydrolysis was carried on by either pepsin or trypsin, although they showed that the products from acid digestion were excellent protein spacers. Numerous experiments in this direction have been carried on by Abderhalden and his pupils.¹² He has worked with both mice and dogs. His earlier experiments tended to show that a protein digested with an enzyme until the biuret reaction was absent could take the place of protein in the diet, but that the acid hydrolytic products could not. Later Abderhalden¹³ brought forth evidence to show that nitrogen equilibrium could be maintained and a retention occur when the products of acid hydrolysis alone were used. With an artificial mixture of amino-acids he was also able to secure nitrogen equilib-

⁴ *Feeds and Feeding.*

⁵ *The Feeding of Animals.*

⁶ *Zeitschr. f. physiol. Chem.*, xlix, p. 64, 1906.

⁷ *Ibid.*, xxxiv, p. 527, 1901.

⁸ *Ibid.*, li, p. 384, 1907.

⁹ *Arch. f. exp. Path. u. Pharm.*, xlviii, p. 303, 1902.

¹⁰ *Amer. Journ. of Physiol.*, ix, p. 386, 1903.

¹¹ *Zeitschr. f. physiol. Chem.*, xlix, p. 113, 1906.

¹² *Ibid.*, xlii, p. 528, 1904.

¹³ *Ibid.*, lxxvii, p. 23, 1912.

rium¹⁴ and effect a partial nitrogen retention. Abderhalden¹⁵ has even furnished experimental evidence, although not very convincing, that with a diet rich in carbohydrates and fat, he was able to secure nitrogen retention when the sole source of nitrogen in the diet was an ammonium salt. This work was done with dogs.

From the above experimental work it is apparent that the simple amino-acids have direct nutritive value and very probably represent a large proportion of the normal end products of protein digestion. The work of Bergmann,¹⁶ Howell¹⁷ and Folin and Denis¹⁸ would indicate that they are absorbed into the blood and used directly for cell repair and protein synthesis.

The fact that our hays and root crops may contain a certain proportion of nitrogen in true amide form, as in asparagine or glutamine, raises the question of the nutritive value of their nitrogen. The part of it which is in the α -amino-position must possess that value which can be assigned to amino-acid nitrogen. As early as 1894, Weiske¹⁹ showed that for herbivora asparagine was a very effective protein sparer. Zuntz²⁰ had suggested that the "amide nitrogen" is built into protein by the aid of intestinal bacteria before it becomes utilizable by these animals. This view was also shared by Müller,²¹ who has shown that the intestinal bacteria could form protein from asparagine and also from ammonium tartrate. Schulze²² was also of the opinion that this is the way in which the amides are useful to herbivora. On the other hand Morgen,²³ working with sheep, has shown that there was not a building of indigestible protein by the intestinal bacteria when ammonium acetate and asparagine were fed. This view does not prove or disprove the possibility of the formation of bacterial proteins as intermediary bodies in the utilization of ammonium

¹⁴ *Zeitschr. f. physiol. Chem.*, lxxvii, p. 23, 1912.

¹⁵ *Ibid.*, lxxviii, p. 1, 1912.

¹⁶ *Beitr. z. chem. Physiol. u. Path.*, vi, p. 40, 1905.

¹⁷ *Amer. Journ. of Physiol.*, xvii, p. 273, 1906.

¹⁸ *This Journal*, xi, p. 87, 1912.

¹⁹ *Zeitschr. f. Biol.*, xxx, p. 254, 1894.

²⁰ *Arch. f. d. ges. Physiol.*, xlix, p. 477, 1891.

²¹ *Ibid.*, cxii, p. 245, 1906.

²² *Zeitschr. f. physiol. Chem.*, lvii, p. 67, 1908.

²³ *Landw. Versuchsstat.*, lxxiii, p. 283, 1912.

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salts and asparagine by herbivora. Morgen and his associates have shown further that the non-protein nitrogen, as in ammonium salts or asparagine, besides being capable of supporting maintenance, could also contribute to the production of milk proteins. Kellner,²⁴ experimenting with lambs, concluded that asparagine and ammonium salts were capable of aiding in the maintenance of nitrogen equilibrium, but were incapable of conversion into body protein.

The evidence from the literature cited above appears to show that with herbivora the "amides," or non-protein nitrogen, can serve to maintain the protein tissues of the body and there is certain evidence that they can also support milk production. Evidence is lacking that growth can be produced.

With rats Politis²⁵ was unable to secure any evidence of protein sparing by asparagine when the latter was the sole source of nitrogen. The same result was obtained by Henriques and Hanson²⁶ but, when the latter used the expressed juice of etiolated beans or peas, they found that a sparing action was partly effected. The "amide nitrogen" from potatoes or roots was ineffective as a protein sparer when fed to rats. Luthje²⁷ showed the same thing to be true with rabbits when the sole source of nitrogen was the protein-free "amide" material obtained from fresh potatoes. Voltz²⁸ concluded that herbivora can make use of "amide nitrogen" and the nitrogen in ammonium salts and he has lately²⁹ reached the same conclusion for carnivora.

Evidently the kind of animal is an important matter in determining how effective these simple nitrogenous substances may be for both maintenance and growth but this is probably more a matter of individual temperament and influence on appetite than of an actual difference in the nutrition processes under normal conditions. In the literature the idea prevails that herbivora can apparently make more efficient use of these simple nitrog-

²⁴ *Arch. f. d. ges. Physiol.*, cxiii, p. 480, 1906; *Zeitschr. f. Biol.*, xxxix, p. 313, 1900.

²⁵ *Zeitschr. f. Biol.*, xxviii, p. 492, 1891.

²⁶ *Zeitschr. f. physiol. Chem.*, liv, p. 169, 1907.

²⁷ *Arch. f. d. ges. Physiol.*, cxiii, p. 547, 1906.

²⁸ *Ibid.*, cxvii, p. 541, 1907.

²⁹ *Zeitschr. f. physiol. Chem.*, lxxix, p. 415, 1912.

enous substances than the omnivora or carnivora. Yet Abderhalden claims to have caused nitrogen retention in a dog with an artificial mixture of amino-acids and even with ammonium salts. One trouble in the feeding of concentrated plant extracts is that they usually lead to intestinal derangement and diarrhea follows. This may be expected from the large salt content of such concentrates.

Plan of experiment.

The plan of our experiment was to secure experimental data on the rate of nitrogen retention by growing heifers when the source of the nitrogen in the ration was mainly either the corn grain or the whole alfalfa plant. The corn kernel contains only traces of "amide nitrogen" and would serve as an excellent check on the alfalfa periods. The corn ration consisted of corn meal, gluten feed and corn stover. The gluten feed was added for the purpose of bringing the nitrogen content of the ration up to a good growing level. It is necessary with this class of animals to use some roughage and, as the corn stover introduced very little available nitrogen and at the same time would maintain an alkaline urine, it was used. The alfalfa ration consisted mainly of alfalfa hay and corn starch. Part of the alfalfa was ground in order that the bulk of the material might be reduced and at the same time a small amount of corn stover was introduced. It was believed that the corn stover would help consumption, but later experiments have shown that this is unnecessary. The starch was used for the purpose of making the net available energy of the two rations closely comparable. It was believed that, should the rate of nitrogen retention on the two rations through a long period of observation be approximately alike, we would have substantial evidence that the "amide nitrogen" was being used for growth. We would certainly have evidence whether the total nitrogen from one source and in the proportion used was as efficient for growth as from the other source. And for us this is an important point.

Two animals were used in the experiment, one receiving the corn ration while the other received the alfalfa ration. After six or eight weeks' observation, the rations were changed and the animal originally receiving the corn ration went to the alfalfa

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ration and *vice versa*. This would obviate any differences in individual powers of growth or capacity for utilization, which is an important factor in such experiments.

A record of income and outgo of nitrogen was quantitatively kept, the feces, urine, etc., being quantitatively collected, sampled and analyzed. With the ration, salt and water were fed *ad libitum*. Each animal was fed such portions of the ration as would be entirely consumed. At a definite time of the day, weekly weighings of the animals were made.

Experiment in 1910-1911.

Two young growing heifers, of approximately 400 to 500 pounds weight, were chosen. They were grade Holsteins, number 1 weighing 505 pounds and number 2, 440 pounds. The rations fed are shown in table I. These figures are taken from Henry's Feeds and Feeding and Armsby's Tables for Production Therms. This table simply shows the proportion in which the materials in an air dried condition were mixed and the approximate likeness of the two rations in production therms, nutritive ratio and digestible protein. This last figure is, of course, based on the total nitrogen and includes the "amide nitrogen." The table also includes in the last column the actual grams of nitrogen contained in such weights of the feed as are indicated in column 2.

It should be noted that the nutritive ratio of the alfalfa ration was 1:9. If the "amide nitrogen" is not included, but only true protein made the basis of the calculation, then the nutritive ratio becomes 1:12.4, a very wide ratio for growth.

From table I it will be seen that on the basis of average analyses, the two rations were very nearly alike in total digestible protein and available therms. From actual nitrogen determinations, the total nitrogen intake in the two rations was also very closely alike. With the consumption of approximately equal quantities of air dried material, the intake of total digestible nitrogen, as well as total nitrogen, was substantially identical in both rations. We had no difficulty in securing a liberal consumption of the rations and approximately equal portions by either animal. The daily consumption for the entire sixteen weeks varied from 7 to 10 pounds.

TABLE I.
Composition of materials used.

	POUNDS	DIGESTIBLE NUTRIENTS			PRODUCTION THERMS	NUTRI- TIVE RATIO	TOTAL N
		Protein	Carbo- hydrate	Fat			
		pounds	pounds	pounds			grams
Corn meal.....	5	0.40	3.33	0.22	4.44		32.46
Gluten feed.....	2	0.41	0.97	0.17	1.58	1:8.2	39.04
Corn stover.....	7	0.12	2.26	0.05	1.85		34.95
Total.....	14	0.93	6.56	0.44	7.87		106.45
Corn stover.....	3	0.05	0.97	0.02	0.79		14.98
Alfalfa hay.....	5	0.88	3.16	0.09	2.75	1:9.0	56.97
Alfalfa meal.....	3						34.21
Starch.....	4.2	0.00	4.00	0.00	4.20		1.52
Total.....	15.2	0.93	8.13	0.11	7.74		107.68

In all, sixteen weeks of actual observation are involved in this first experiment, 1910-1911. Before the records were begun there was a preliminary period of ten days' feeding. In period I, of eight weeks, animal 1 received the corn ration and animal 2 the alfalfa ration. At the end of that period the rations were changed. This was done slowly and by substituting a pound of one ration for a pound of the other, consuming in all eight days for the transfer. In period II, animal 1 received the alfalfa ration, while animal 2 received the corn ration. In tables II and III are included the records of this first experiment. The data are condensed from daily records to weekly composites and represent in grams the nitrogen consumed per week and the nitrogen excreted and retained. In addition are columns showing the pounds of feed consumed and a record of the gain in live weight.

Animal 1 stored 451.7 grams of nitrogen during the corn period or 13.4 per cent of the total intake and 754.2 grams on the alfalfa ration or 20.4 per cent of the total intake. It will be noted that there is considerable oscillation in the weekly records of the amounts of nitrogen retained. This is illustrated in charts I and II, which are graphic records of nitrogen retention and nitrogen consumption. It is very doubtful if this means a variation in the rates of cell growth and nitrogen storage but, rather, a differ-

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TABLE II.

Record of balance of income, outgo and retention of nitrogen, feed consumed and gain in live weight.

Animal 1. Period I. Corn ration.

DATE 1910-1911	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL, 505 LBS.)
	pounds	grams	grams	grams	grams	pounds
Dec. 2-8	8	425.8	182.4	186.9	55.5	513
9-15	7-8	418.2	199.2	200.0	19.0	521
16-22	7	380.9	168.6	178.5	33.8	493
23-29	8-9	430.7	175.3	173.0	82.4	509
Jan. 30-5	8	425.8	190.9	161.7	73.2	520
6-12	8	409.9*	185.4	163.1	61.4	524
13-19	9	431.2	222.7	169.0	39.5	531
20-26	9	431.2	204.9	139.4	86.9	534
					451.7	

Animal 1. Period II. Alfalfa ration.

Feb. 4-10	9	427.4	187.6	152.4	87.4	544
11-17	9	427.4	184.0	186.7	56.7	544
18-24	9-10	454.6	187.8	148.4	108.4	556
25-3	10	474.6	208.8	164.2	100.2	576
Mar. 4-10	10	474.6	218.6	155.8	100.2	575
11-17	10	474.6	214.9	144.1	115.6	579
18-24	10	474.6	220.0	170.4	84.2	581
25-31	10	474.6	217.7	155.4	101.5	592
					754.2	

* New corn stover used; 7 pounds contained 24.33 grams of N.

ence in the amounts of nitrogen excreted, as determined by the artificial periods of observation prescribed. The efficiency of the total alfalfa nitrogen for growth was for animal 1 somewhat greater than that of the corn kernel. While we do not care to lay too much stress on the positive figures for alfalfa obtained in this case, nevertheless, the data show that the total nitrogen from this plant was not inferior for growth to that from the corn kernel. In the transition period from the corn to the alfalfa ration, there was no sudden rise in the nitrogen output, either in the feces or urine. The ingestion of a large proportion of "amide nitrogen" with the change to the alfalfa ration, should have led to such increased excretion had this not been involved in the normal

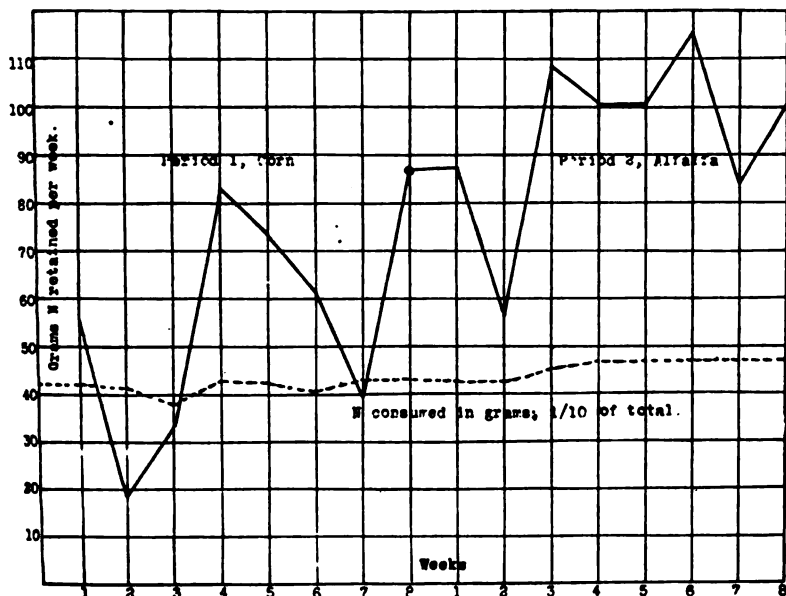


CHART I, ANIMAL 1. CURVE OF N RETENTION (UNBROKEN LINE). CURVE OF N CONSUMPTION (DOTTED LINE).

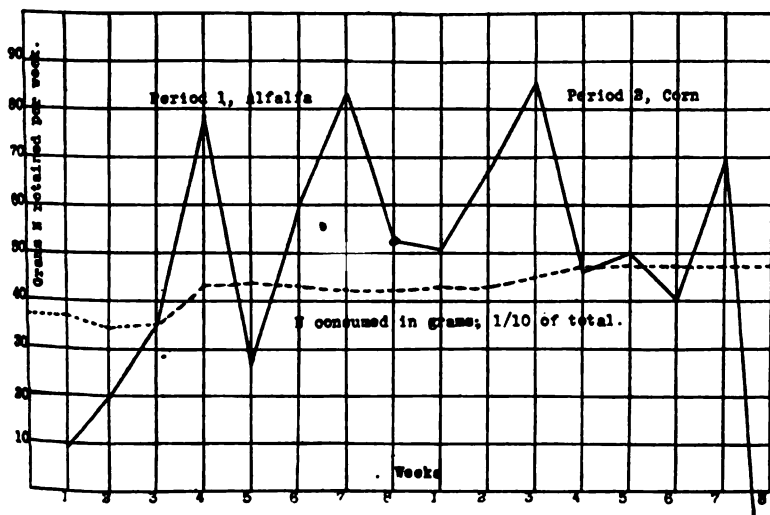


CHART II, ANIMAL 2. CURVE OF N RETENTION (UNBROKEN LINE). CURVE OF N CONSUMPTION (DOTTED LINE).

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TABLE III.

Record of balance of income, outgo and retention of nitrogen, feed consumed and gain in live weight.

Animal 2. Period I. Alfalfa ration.

DATE 1910-1911	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL, 440 LBS.)
	<i>pounds</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>pounds</i>
Dec. 2-8	7-8	375.4	167.6	198.0	9.8	445
9-15	7	347.1	149.1	178.0	20.0	426
16-22	7-8	354.0	132.5	186.4	35.1	430
23-29	8-9	439.2	181.3	178.4	79.5	447
Jan. 30-5	9	446.3	211.0	207.9	27.4	452
6-12	9	430.2*	206.0	163.9	60.3	453
13-19	9	427.5	208.9	134.9	83.7	456
20-26	9	427.5	197.2	177.5	52.8	454
					368.6	

Animal 2. Period II. Corn ration.

Feb. 4-10	9	431.2	191.3	188.5	51.4	480
11-17	9	431.2	203.3	160.2	67.5	481
18-24	9-10	458.6	231.6	141.1	85.9	502
25-3	10	479.2	239.6	192.8	46.8	508.
Mar. 4-10	10	479.2	257.4	171.9	49.9	522
11-17	10	479.2	247.0	191.7	40.5	514
18-24	10	479.2	219.1	190.3	69.8	527
25-31	10	479.2	271.6	249.2	-41.4	525
					369.4	

* New corn stover used; 7 pounds contained 24.33 grams of N.

processes of nitrogen utilization and elimination. Of the total nitrogen absorbed, animal 1 retained* during the corn period 24.7 per cent and during the alfalfa period 36.9 per cent.

Animal 2 stored 368.6 grams of nitrogen during the alfalfa period or 11.3 per cent of the total intake and 369.4 grams on the corn ration or 9.9 per cent. The same oscillation in weekly storage is also to be observed with this animal. Apparently animal 2 did not possess the same powers of growth as animal 1. In neither period was the amount stored as great as in the case of animal 1. At the end of period II, this animal suffered somewhat from diarrhea, a fact which accounts for the negative balance of that period. With this animal the amount of nitrogen retention on

the two rations was practically alike, indicating again no inferiority of the alfalfa nitrogen for processes of growth.

Again in this case there was no sudden decrease of nitrogen in the feces or urine as the change from the alfalfa ration to the corn ration was made. During the alfalfa period animal 2 stored 20.5 per cent of the absorbed nitrogen and 18.8 per cent during the corn period.

Attention should be called to the discrepancy in the live weight gains as compared with actual nitrogen storage. Animal 1 gained in gross live weight during the sixteen weeks some 79 pounds, with an actual storage of 1205.9 grams of nitrogen. Animal 2 gained 80 pounds in live weight during the same period, but actually stored but 738 grams of nitrogen or approximately three-fifths as much as animal 1.

Experiment in 1911-1912.

The plans of experimentation in 1911-1912 were substantially those of the previous year. Two young growing heifers of grade Holstein breeding and about 300 to 400 pounds in weight were again chosen. The rations used were of the same kind of materials as in the previous experiment, except that no corn stover was introduced into the alfalfa ration. This ration consisted wholly of alfalfa and starch. Again a part of the alfalfa hay was reduced to meal in our own grinders for the purpose of reducing the bulk. The composition of the ration is appended in table IV.

It will be seen that the rations were very similar in respect to total digestible nutrients, therms and nutritive ratio. The total nitrogen intake with like weight of air dried mixture was also substantially identical.

In this experiment there was a preliminary three weeks' feeding period with a low nitrogen ration. This ration consisted of 4 pounds of wheat straw, 3 pounds of corn starch, 0.2 pound of cane sugar and 1 ounce of calcium phosphate. Salt was given freely. This preliminary period was to determine, on as low a nitrogen intake as practicable with the materials available, what the creatinine output would be and the relation of the creatinine nitrogen to the total nitrogen of the urine. McCollum³⁰ has shown, with

³⁰ Research Bulletin 21, Wisconsin Exp. Sta.

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TABLE IV.
Composition of materials used.

	FEED	DIGESTIBLE NUTRIENTS			PRODUC- TION THERMS	NUTRITIVE RATIO	TOTAL N
		Protein	Carbo- hydrate	Fat			
	pounds	pounds	pounds	pounds			grams
Corn meal.....	5	0.40	3.33	0.22			33.36
Gluten feed.....	2	0.41	0.97	0.17			40.42
Corn stover.....	7	0.12	2.26	0.05			37.67
Total.....	14	0.93	6.56	0.44	7.8	1:8.2	111.45
Alfalfa hay.....	5						60.33
Alfalfa meal.....	4	0.99	3.51	0.10			51.33
Starch.....	5		5.00				1.36
Total.....	14	0.99	8.51	0.10	8.0	1:8.5	113.02

pigs on practically nitrogen-free rations but with the energy requirement satisfied, that the creatinine excretion reaches a maximum and is a fairly definite proportion of the total urinary nitrogen. This would indicate that it was a constant end product of endogenous metabolism. Further he has shown that with increasing nitrogen retention in pigs there is a fairly definite proportional increase in the creatinine output. This would indicate that creatinine would serve as an index of protein storage. It was to learn whether such quantitative relationships held for herbivora that we instituted this preliminary feeding period.

Effect of low nitrogen intake on creatinine excretion. In table V are given the data for this preliminary three weeks' period, with both animals, on the effect of low nitrogen intake on creatinine output. This is expressed in grams and the per cent of creatinine nitrogen of the total urinary nitrogen. A week of feeding preceded the taking of these records.

In animal 3 the proportion of the total nitrogen of the urine as creatinine nitrogen rose as high as McCollum has reported for pigs receiving nitrogen-free rations, and on single days it went as high as 25 per cent. With animal 4 the ratio did not rise as high, although in the first week it was approximately 20 per cent. After the first week it decreased in both cases, possibly due to the losses of body protein incident to the low nitrogen ration. The average

TABLE V.

*Record of low nitrogen feeding and creatinine output.**Animal 3.*

DATE	N INTAKE	N IN FECES	N IN URINE	N LOSS	DAILY AVERAGE CREATININE	CREATININE N AS AVERAGE PERCENTAGE OF TOTAL URI- NARY N
	grams	grams	grams	grams	grams	per cent
Dec. 12-18	39.48	59.97	43.73	64.2	3.67	22.0
19-25	39.48	63.66	34.11	58.2	2.71	21.0
26-Jan. 1	39.48	66.50	36.26	63.2	2.14	15.2

Animal 4.

Dec. 12-18	41.09	78.97	47.85	85.7	3.39	18.4
19-25	41.09	79.75	51.88	90.5	2.55	11.9
26-Jan. 1	41.09	67.61	33.28	59.8	1.61	12.9

daily grams of creatinine excreted were also highest in the first week in the case of both animals and decreased perceptibly under nitrogen starvation. It should be noted that this high ratio of creatinine nitrogen to total urinary nitrogen was reached in the first week of the record and the second week of feeding. This high ratio was reached somewhat earlier than was observed by McCollum with pigs on nitrogen-free diets.

We shall discuss the creatinine rise on our growing ration further on in this paper.

After the preliminary feeding the animals were immediately placed on the growing rations of corn and alfalfa. They were to receive these rations for six weeks and then be immediately changed to the ration of the other. It is very essential that this should be done as then any individual differences in powers of growth are obviated.

The records of nitrogen income, outgo and storage, as well as columns showing the food consumption and increase in live weight, are given in tables VI and VII. Nitrogen records are in grams per week and are seven-day composites of the daily record.

Animal 3 stored during the alfalfa period 592.2 grams of nitrogen or 28.7 per cent of the total intake. During the corn period 615.6 grams of nitrogen were stored or 24.1 per cent of the total nitrogen consumed. During the first week of record on the alfalfa

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ration 45 per cent of the total nitrogen ingested was stored. This very high efficiency is to be attributed to the partial nitrogen depletion the animal had suffered during the preliminary period of partial nitrogen starvation, but even after the first week there was a continued high efficiency of storage, which was maintained on both rations for the entire period of record. This is in marked contrast to animals 1 and 2 where the storage was not much over 10 per cent of the total nitrogen ingested. Both animals 3 and 4 were somewhat younger and lighter in weight than 1 and 2, which are additional factors in the rate of growth.

The data show that the nitrogen in the alfalfa ration under the conditions of the experiment is just as efficient for growth as that of the corn kernel. Of the nitrogen absorbed from the intestine during the alfalfa period 48 per cent was retained while during the corn period 40 per cent of the absorbed nitrogen remained in the body. The sudden change from the alfalfa ration to the corn

TABLE VI.

Record of income, outgo, retention of nitrogen, feed consumed and gain in live weight.

Animal 3. Period I. Alfalfa ration.

DATE	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL, 345 LBS.)
	pounds	grams	grams	grams	grams	pounds
Jan. 2-8	6-7	339.0	114.5	71.0	153.5	347
9-15	6-7	339.0	143.3	87.0	108.7	343
16-22	6-7	339.0	133.5	111.6	93.9	348
23-29	6-7	339.0	133.7	119.3	86.0	352
30-Feb. 5	6-7	363.2	155.0	122.4	85.6	356
Feb. 6-12	6-7	339.0	153.7	120.8	64.5	358
					592.2	

Animal 3. Period II. Corn ration.

Feb. 13-19	6-7	358.2	140.4	151.1	66.7	372
20-26	7	413.9	163.6	118.9	131.4	386
27-Mar. 4	7-8	445.7	196.0	122.7	127.0	393
Mar. 5-11	7-8	445.7	176.5	149.3	119.9	401
12-18	7-8	445.7	170.4	179.2	96.1	404
19-25	7-8	445.7	183.9	187.3	74.5	415
					615.6	

ration should have materially decreased the nitrogen in the urine if it were true that practically one-fourth of the nitrogen of the alfalfa was in useless form and unsuited for building purposes. The hypothesis that there is formed in the digestive tract from the "amide nitrogen" indigestible bacterial proteins also does not receive support from our data. In the two weeks before and after the change in ration the nitrogen content of the feces is practically identical. Apparently the two sources of nitrogen are being handled in identical fashion.

Animal 4 stored during the corn period 879.3 grams of nitrogen or 35.4 per cent of the total intake. During the alfalfa period 822.8 grams were stored or 29.6 per cent of the total nitrogen ingested. Here, again, as with animal number 3, the first week showed a very high nitrogen retention, amounting to 44 per cent of the total intake. This followed immediately a period of nitrogen starvation and accounts for such high efficiency. This com-

TABLE VII.

Record of income, outgo, retention of nitrogen, feed consumed and gain in live weight.

Animal 4. Period I. Corn ration.

DATE	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL, 387 LBS.)
	pounds	grams	grams	grams	grams	pounds
Jan. 2-8	6-7	342.2	112.1	77.2	152.9	379
9-15	7	390.0	134.6	131.7	123.7	382
16-22	7	390.0	147.3	118.5	124.2	385
23-29	8	451.9	161.5	103.4	187.0	400
30-Feb. 5	8	451.9	173.9	125.5	142.5	407
6-12	8	451.9	166.4	126.5	149.0	415
					879.3	

Animal 4. Period II. Alfalfa ration.

Feb. 13-19	7-8	443.8	169.9	113.8	160.1	424
20-26	8-9	476.1	174.1	123.6	178.4	435
27-4	9	492.2	194.6	135.5	162.1	440
Mar. 5-11	8	451.9	182.8	185.4	83.7	430
12-18	8	451.9	156.7	171.0	124.2	442
19-25	8	451.9	169.6	168.0	114.3	432
					822.8	

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paratively high retention continued during both periods of feeding and was practically parallel with that of animal 3. During the corn period 55 per cent of the total nitrogen absorbed from the intestine was retained, while in the alfalfa period 48 per cent was retained. With both animals there was a slow decrease in the rate of nitrogen retention as the periods progressed. When the rations were suddenly changed there was in both cases an increase in the rate of nitrogen retention for a two weeks' period and then a decrease. Such variations are difficult to explain and at present can only be interpreted on the basis of lags in elimination. Such rises and falls may occur any time in the period of feeding as illustrated in charts III and IV, which show the curves of nitrogen

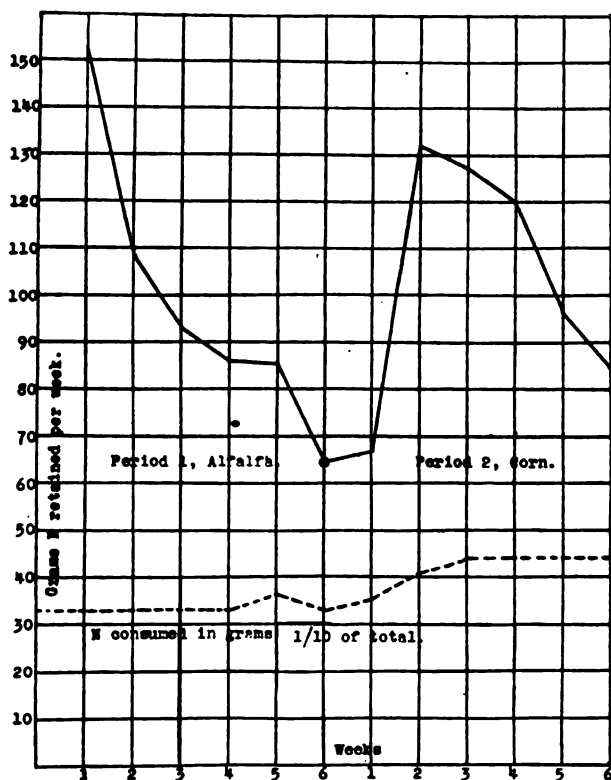


CHART III, ANIMAL 3. CURVE OF N RETENTION (UNBROKEN LINE). CURVE OF N CONSUMPTION (DOTTED LINE).

retention and consumption for animals 3 and 4. Animal 4 gives a clear-cut demonstration of the failure of a sudden change from the corn to the alfalfa ration to raise the nitrogen excretion in either urine or feces. Even for two weeks, which is beyond the range of a lag of elimination, the nitrogen in the feces and urine did not rise perceptibly above what it was in the last week on the corn ration.

At the end of this period of feeding, both animals were again placed upon the nitrogen-low ration of straw and starch for a period of ten days for the purpose of again determining the ratio of creatinine nitrogen to the total urinary nitrogen. In the case

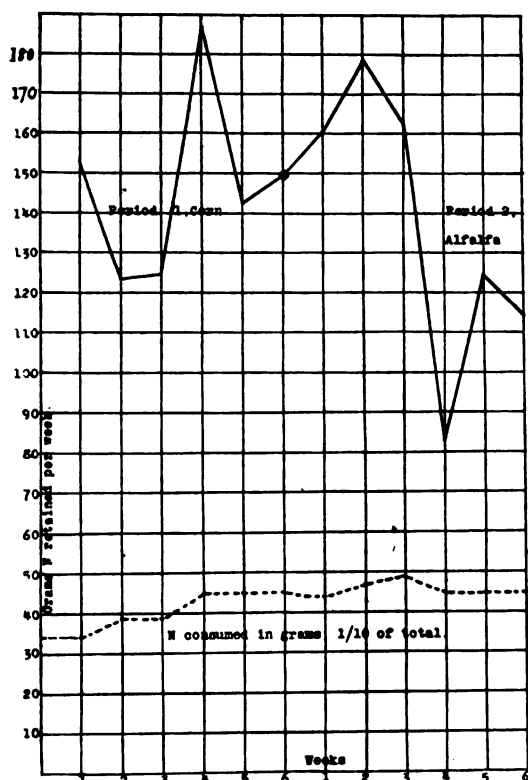


CHART IV, ANIMAL 4. CURVE OF N RETENTION (UNBROKEN LINE). CURVE OF N CONSUMPTION (DOTTED LINE).

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of animal 3 this rose on March 31 to 25.7 per cent and remained near this figure until the close of the record, April 3. Animal 4 did not show such a high relation, reaching 17.7 per cent on March 31 and 19 per cent on April 3.

Reference to table VIII shows that during the first three weeks of observation on a nitrogen-low ration the average daily creatinine excretion decreased from 3.67 grams to 2.14 grams in animal 3 and from 3.39 to 1.61 grams for animal 4. This decrease was possibly due to protein losses from the body. In table VIII is recorded the average daily creatinine excretion in grams for the entire period of experimental feeding.

It is apparent from the data that for a time there was a steady rise of creatinine output accompanying nitrogen storage. With animal 4 this reached a maximum a considerable number of weeks before the end of the experiment and while she was still growing and storing nitrogen. Animal 3 rose to a high output on the week of February 27 to March 14, then decreased and rose again show-

TABLE VIII.

Record of creatinine excretion during the entire period of feeding.

Animal 3.	Animal 4.
1910-11	1910-11
1911-12	1911-12
1912-13	1912-13
1913-14	1913-14
1914-15	1914-15
1915-16	1915-16
1916-17	1916-17
1917-18	1917-18
1918-19	1918-19
1919-20	1919-20
1920-21	1920-21
1921-22	1921-22
1922-23	1922-23
1923-24	1923-24
1924-25	1924-25
1925-26	1925-26
1926-27	1926-27
1927-28	1927-28
1928-29	1928-29
1929-30	1929-30
1930-31	1930-31
1931-32	1931-32
1932-33	1932-33
1933-34	1933-34
1934-35	1934-35
1935-36	1935-36
1936-37	1936-37
1937-38	1937-38
1938-39	1938-39
1939-40	1939-40
1940-41	1940-41
1941-42	1941-42
1942-43	1942-43
1943-44	1943-44
1944-45	1944-45
1945-46	1945-46
1946-47	1946-47
1947-48	1947-48
1948-49	1948-49
1949-50	1949-50
1950-51	1950-51
1951-52	1951-52
1952-53	1952-53
1953-54	1953-54
1954-55	1954-55
1955-56	1955-56
1956-57	1956-57
1957-58	1957-58
1958-59	1958-59
1959-60	1959-60
1960-61	1960-61
1961-62	1961-62
1962-63	1962-63
1963-64	1963-64
1964-65	1964-65
1965-66	1965-66
1966-67	1966-67
1967-68	1967-68
1968-69	1968-69
1969-70	1969-70
1970-71	1970-71
1971-72	1971-72
1972-73	1972-73
1973-74	1973-74
1974-75	1974-75
1975-76	1975-76
1976-77	1976-77
1977-78	1977-78
1978-79	1978-79
1979-80	1979-80
1980-81	1980-81
1981-82	1981-82
1982-83	1982-83
1983-84	1983-84
1984-85	1984-85
1985-86	1985-86
1986-87	1986-87
1987-88	1987-88
1988-89	1988-89
1989-90	1989-90
1990-91	1990-91
1991-92	1991-92
1992-93	1992-93
1993-94	1993-94
1994-95	1994-95
1995-96	1995-96
1996-97	1996-97
1997-98	1997-98
1998-99	1998-99
1999-00	1999-00
2000-01	2000-01
2001-02	2001-02
2002-03	2002-03
2003-04	2003-04
2004-05	2004-05
2005-06	2005-06
2006-07	2006-07
2007-08	2007-08
2008-09	2008-09
2009-10	2009-10
2010-11	2010-11
2011-12	2011-12
2012-13	2012-13
2013-14	2013-14
2014-15	2014-15
2015-16	2015-16
2016-17	2016-17
2017-18	2017-18
2018-19	2018-19
2019-20	2019-20
2020-21	2020-21
2021-22	2021-22
2022-23	2022-23
2023-24	2023-24
2024-25	2024-25
2025-26	2025-26
2026-27	2026-27
2027-28	2027-28
2028-29	2028-29
2029-30	2029-30
2030-31	2030-31
2031-32	2031-32
2032-33	2032-33
2033-34	2033-34
2034-35	2034-35
2035-36	2035-36
2036-37	2036-37
2037-38	2037-38
2038-39	2038-39
2039-40	2039-40
2040-41	2040-41
2041-42	2041-42
2042-43	2042-43
2043-44	2043-44
2044-45	2044-45
2045-46	2045-46
2046-47	2046-47
2047-48	2047-48
2048-49	2048-49
2049-50	2049-50
2050-51	2050-51
2051-52	2051-52
2052-53	2052-53
2053-54</	

DATE	FEED	AVERAGE DAILY CREATININE	DATE	FEED	AVERAGE DAILY CREATININE
		grams			grams
Dec. 12-18	{ Starch- straw period }	3.67	Dec. 12-18	{ Starch- straw period }	3.39
19-25		2.71	19-25		2.55
26-Jan. 1		2.14	26-Jan. 1		1.61
Jan. 2-8	Alfalfa	2.47	Jan. 2-8	Corn	2.78
9-15	Alfalfa	2.74	9-15	Corn	2.77
16-22	Alfalfa	2.75	16-22	Corn	2.64
23-29	Alfalfa	3.28	23-29	Corn	3.36
30-Feb. 5	Alfalfa	3.68	30-Feb. 5	Corn	4.13
Feb. 6-12	Alfalfa	3.74	Feb. 6-12	Corn	4.28
13-19	Corn	4.11	13-19	Alfalfa	4.44
20-26	Corn	4.10	20-26	Alfalfa	4.20
27-Mar. 4	Corn	4.21	27-Mar. 4	Alfalfa	4.33
Mar. 5-11	Corn	3.79	Mar. 5-11	Alfalfa	4.10
12-18	Corn	3.38	12-18	Alfalfa	3.14
19-25	Corn	4.25	19-25	Alfalfa	4.15
25-Apr. 3	{ Starch- straw period }	4.45	25-Apr. 3	{ Starch- straw period }	4.18

ing a maximum in the last ten days or during the starch-straw period. As a product of endogenous metabolism it should rise in the amount excreted accompanying protein storage, if there were no agents of destruction or if it can be looked upon as a quantitative end product of endogenous metabolism. According to Gottlieb and Stangassinger³¹ both creatinine and creatine are destroyed by tissue enzymes, but Mellanby³² denies this. In experiments with five pigs, McCollum³³ obtained in two cases very satisfactory data on the quantitative relation of the rise of creatinine output with nitrogen storage although with the other animals³⁴ he was unable to secure such concordant data. This work will be reported in detail later, as well as a further discussion of the factors affecting creatinine excretion. It is very apparent that with heifers the rise of creatinine excretion is not a quantitative accompaniment of protein storage and unfortunately cannot be made the index for measuring the efficiency for growth of various rations. Apparently the amount of creatinine excreted in the urine is the total creatinine of endogenous metabolism minus that which has been destroyed in the tissues.

GENERAL DISCUSSION.

In experiments of this character too much stress cannot be laid on the importance of maintaining a normal condition of the animal and of continuing the experiment over a long period of time. The weakness of so many experiments dealing with this question of the relative value of "amide nitrogen" has been due to an attempt to feed an extract of plant tissue or a pure compound such as asparagine. The first invariably leads to the production of diarrhea and the second does not represent the status of a large part of the "amide nitrogen" of plant tissue. In our work we have made use of a method of comparison using the same animal for one period, on a ration the protein of which was almost wholly made up of true digestible protein of a cereal grain, and for the second period on the digestible protein of a legume hay which includes the "amide nitrogen." The nutritive ratios of the two rations,

³¹ *Zeitschr. f. physiol. Chem.*, lii, p. 14, 1900.

³² *Journ. of Physiol.*, xxxvi, p. 461. 1908.

³³ Research Bulletin 21, Wisconsin Exp. Sta.

³⁴ *This Journal*, xi, p. 15, 1912.

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based on crude digestible protein, were substantially identical; but if in the case of the hay the "amide nitrogen" be excluded then the nutritive ratio becomes 1:12.4. The efficiency of the two rations for growth, based on nitrogen storage, was essentially the same. It may be urged that in the alfalfa ration there was sufficient true protein to furnish all the nitrogen storage observed and that there is no proof that the "amide nitrogen" took part in supplying nitrogen for such storage. There are no records, so far as we are aware, of the efficiency of a 1:12.4 nutritive ratio from any source for growth and nitrogen storage, but it is the experience of feeders that such a ratio will not lead to rapid growth. As the outgrowth of very much experience the use of such a ratio is certainly not the practice. We must either believe that a nutritive ratio of 1:12.4 with alfalfa hay is as efficient for nitrogen storage as a 1:8.2, as used in the corn ration, or that the "amide nitrogen" has been used for protein synthesis. Moreover, the failure of the "amide nitrogen" to increase or decrease the fecal or urinary nitrogen, when the ration was suddenly changed, is very strong evidence that that nitrogen was being used in ways similar to that of the nitrogen of true protein origin.

Attention should also be called to the very great wastefulness of nitrogen by these growing animals. With liberal energy and carbohydrate intake, their power to retain nitrogen for growth was really small. Animal 4 for example in the second period of observation—a period of forty-two days—received some 2700 grams of nitrogen, of which at least 1700 grams were absorbed from the intestine. Yet of this 1700 grams absorbed, only 822 grams were retained. Such extravagant waste must bear some relation to the narrow nutritive ratio or high nitrogen intake necessary for efficient growth. Obviously, if we accept the prevailing hypothesis that the value for growth of ingested protein will depend upon its degree of similarity to the tissue proteins, then such figures become intelligible.

CONCLUSIONS.

This paper includes the records of the comparative efficiency of nitrogen from alfalfa hay and the corn kernel for growth. Also data on the relation of creatinine output to nitrogen storage in heifers.

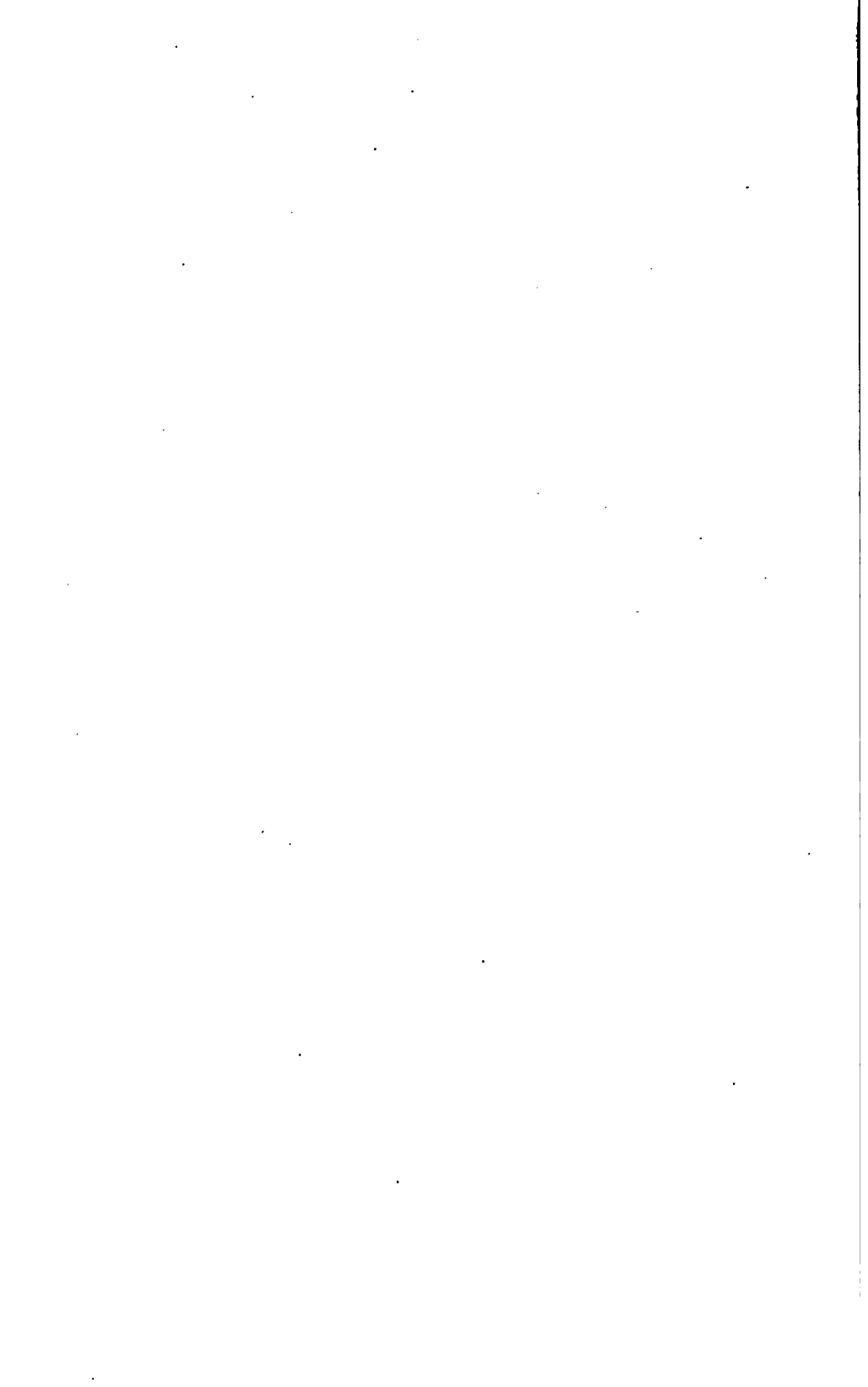
1. On the basis of total nitrogen ingested, the utilization of nitrogen for growth was as efficient when the source was from alfalfa hay as when it came from the corn kernel.

2. With high intake of total digestible crude protein, which in the case of alfalfa includes the "amide nitrogen," the storage of nitrogen was essentially alike on the two rations.

3. There was no sudden decrease or increase in the nitrogen content of the urine or feces when the animals were suddenly changed from one ration to the other. This is evidence that the "amide nitrogen" was being used in the same way as the true protein nitrogen.

4. It is apparent from our data that full value, at least for growth, can be given to the total nitrogen of alfalfa hay. The "amide nitrogen" should not be considered worthless. The influence on milk production will be studied later.

5. With growing heifers there was no very concordant rise in creatinine output with increased storage of nitrogen. This precludes the possibility of using this index for these animals as a measure of the efficiency of a given source of nitrogen to produce nitrogen storage.



ANIMAL CALORIMETRY.

FIFTH PAPER.

THE INFLUENCE OF THE INGESTION OF AMINO-ACIDS UPON METABOLISM.

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(Received for publication, September 14, 1912.)

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I. INTRODUCTION.

The immediate and profound effect of ingesting meat in large quantity was shown and analyzed in the second paper of this series. It will be recalled that Rubner suggested that protein might be of value to the life processes in the organism only in so far as it was convertible into dextrose, the rest of the protein being oxidized with the production of heat which is not available to maintain the motions of cell life. Recent work of the writer¹

¹ Lusk: *Amer. Journ. of Physiol.*, xxii, p. 174, 1908.

and of Ringer and Lusk² has shown that glycocoll and α -alanine are completely converted into dextrose by the phlorhizinized dog, and that three atoms of carbon contained in aspartic and glutamic acids are also convertible into dextrose. These four amino-acids yield about one-half of the sugar derived from protein metabolism in phlorhizin glycosuria.³ This information presented the possibility of testing the validity of Rubner's hypothesis, and as far back as 1908 the writer⁴ gave 40 grams of dextrose to a dog and then 20 grams of dextrose to which 16 grams of alanine were added, and noted no change in the metabolism during twenty-four hours as determined by the Pettenkofer-Voit respiration apparatus. Other similar experiments showed that the addition of 24 grams of tyrosine and of 40 grams of glutamic acid to a standard diet were without effect upon the metabolism of the animal. These experiments have never been published in detail and there is now no reason for such publication. As will be shown in paper six, the action of the amino-acids was masked by the mass action of dextrose or of the standard diet given.

In paper three, it was demonstrated that 20 grams of dextrose administered to Dog II caused in one instance no effect on metabolism, while in another instance it caused a slight increase and this only during the second hour. If 25 grams of glycocoll or 20 grams of alanine which are completely convertible into 20 grams of dextrose in metabolism, behave like dextrose, then, after their ingestion, they should cause only a slight rise in the heat production. If glutamic acid be given it might show a "specific dynamic" action, for three of its carbon atoms are convertible into dextrose while two on oxidation might furnish free heat to the body. Also tyrosine, with its many cleavages before it reaches a stage for use by the cells in metabolism, might show a pronounced effect upon the heat production. The writer freely admits that these were the results which he was prepared to find. Prior to the work here presented, his ideas on this subject had been essentially derived from the teachings of Rubner.

² Ringer and Lusk: *Zeitschr. f. physiol. Chem.*, lxxvi, p. 106, 1910.

³ Lusk: Phlorhizinglukosurie, *Ergeb. d. Physiol.*, xii, p. 315, 1912.

⁴ Lusk: *Proc. Soc. Exp. Biol. and Med.*, vii, p. 136, 1910.

II. EXPERIMENTAL PROCEDURE.

The experiments were conducted as in paper three upon Dog II. The same standard diet was administered at six o'clock in the evening. The amino-acid was dissolved or suspended in 150 cc. of warm water containing 2 grams of Liebig's extract of beef and given at noon.

The total nitrogen and urea plus ammonia nitrogen were determined in hourly periods in the dog on occasions when the meat extract was given, and the animal was not put in the calorimeter. Under similar conditions, total nitrogen and urea plus ammonia nitrogen were determined in hourly periods after an amino-acid had been ingested with the Liebig broth. The difference between the urea plus ammonia nitrogens in the two instances was assumed to represent the amino-acid destruction of the hour. The difference between the total nitrogens of corresponding hours represented approximately the amino-acid metabolized plus the amino-acid eliminated as such. The true protein metabolism of the time was assumed to be the nitrogen which would have been eliminated had no amino-acid or beef extract been administered. From the values thus calculated, approximate figures were obtained which represented the protein and amino-acid metabolism during the hours when the animal was in the calorimeter. A similar procedure was employed with Dog I in paper two when meat was ingested.

In order to determine the indirect calorimetry when amino-acids are metabolized, the table on page 158 was prepared.

III. EXPERIMENTAL PART.

The full analytical details of all the experiments are given in the Appendix, Table I.

A. *Liebig's extract of beef.*

Both Rubner⁵ and Bürgi⁶ have demonstrated that the constituents of Liebig's extract of beef are very largely ready for elimination in the urine. The researches of Pawlow and his pupils

⁵ Rubner: *Zeitschr. f. Biol.*, xix, p. 343, 1883.

⁶ Bürgi: *Archiv für Hygiene*, li, p. 1, 1904.

Physiological values of amino-acids when oxidized in the organism, calculated by deducting the value of urea formed from them in metabolism.

FORMULA	MOLECULAR WEIGHT	PER CENT N	CALORIES				IN OXIDATION OF 1 GRAM OF SUBSTANCE						1 GRAM N =			
			Actual	Physiological		O ₂	CO ₂	O ₂	CO ₂	R. Q.	CALORIC VALUE 1 LITER O ₂	O ₂	CO ₂	C in resp.	grams	grams
			per gram	per gram	per gram N											
Glycocoll.....	C ₂ H ₅ NO ₂	75	18.66*3.110	1.846	9.89	0.64	0.88	0.448	0.448	1.00	4.12	3.43	4.71	1.28		
Alanine.....	C ₃ H ₇ NO ₂	89	15.74*4.401	3.037	19.30	1.08	1.236	0.756	0.629	0.83	4.15	6.86	7.85	2.14		
Glutamic acid.....	C ₅ H ₉ NO ₄	147	9.52*3.662	3.146	33.05	0.98	1.347	0.686	0.686	1.00	4.593	10.32	14.18	3.87		
Leucine.....	C ₆ H ₁₃ NO ₂	131	10.68*6.525	5.946	55.67	1.832	1.840	1.282	0.938	0.73	4.638	17.15	17.23	4.70		
Tyrosine.....	C ₉ H ₁₁ NO ₄	181	7.73*5.915	5.496	71.10	1.679	2.066	1.175	1.054	0.89	4.678	21.72	26.73	7.29		

* Emery and Benedict: *Amer. Journ. of Physiol.*, xxviii, p. 307, 1911. Urea yields 2.528 calories according to these authors which is essentially the same as 2.525, correctly established by Rubner in 1885.

† Stohmann and Langbein: *Journ. f. prakt. Chem.*, xlv, p. 383, 1891.

have shown that meat extracts stimulate a secretory flow of digestive juice in both stomach and pancreas. An experiment by Cohnheim⁷ indicates that the metabolism of a fasting dog may be increased 9 per cent in three hours, when meat which passes out through an oesophageal fistula is given. Such "fictitious feeding" stimulates the flow of both gastric and pancreatic juices. However, Cohnheim made no record of the movements of the dog during the period of observation and these might easily have produced the differences in the heat production reported.

In considering the following results, obtained after giving 2 grams of Liebig's extract of beef in 150 cc. of water, one must remember that the basal metabolism of this dog was shown to be 16.2 calories per hour (paper three). An experiment showing this is here reproduced for comparison with the results after giving Liebig's extract.

The influence of Liebig's extract on metabolism.

TIME	EXP. 21. FEBRUARY 5			EXP. 22. FEBRUARY 6			EXP. 38. MARCH 22		
	NO FOOD			LIEBIG'S EXTRACT			LIEBIG'S EXTRACT		
	Protein	Calories		Protein	Calories		Protein	Calories	
	N	Calc.	Found	N	Calc.	Found	N	Calc.	Found
p.m.	gram			gram			gram		
1.00-2.00	0.094	15.43	15.11	0.136	15.06	16.56	0.142	14.92	15.32
2.00-3.00	0.094	16.03	16.37	0.136	16.37	16.15	0.142	15.44	15.55
3.00-4.00	0.094	18.33	17.35	0.136	17.62	16.95	0.142	16.15	16.56
		49.79	48.83		49.05	49.66		46.51	47.43
Total per hour		16.6			16.4			15.5	

The above table shows an average metabolism of 16 calories per hour after ingesting 2 grams of Liebig's extract in 150 cc. of warm water and it is evident that *Liebig's extract is without influence upon the metabolism in spite of the glandular activity it is known to induce.*

The urinary analyses. The nitrogen content of 2 grams of Liebig's extract of beef was found to be 0.156 gram. After ingestion of this quantity in 150 cc. of water, urinary analyses were made in hourly periods with the following results.

⁷ Cohnheim: *Archiv für Hygiene*, lvii, p. 401, 1906.

Urinary analyses after giving Liebig's extract.

TIME	MARCH 21		MARCH 28	
	TOTAL N	UREA + NH ₃ N	TOTAL N	UREA + NH ₃ N
	gram	gram	gram	gram
12.00-1.00	0.201		0.154	0.152
1.00-2.00	lost		0.169	0.162
2.00-3.00	lost		0.176	0.156
3.00-4.00	0.140	0.121	0.150	0.126
4.00-5.00	0.121	0.104	0.140	0.122
5.00-6.00	0.114	0.104		
Total.....			0.789	0.718

On March 28 the urea plus ammonia N was 91 per cent of the total N.

On the calorimeter days analysis of the urines during periods of four hours and ten minutes showed the following results which are compared with estimated results based on the analysis of the urine of March 28.

DATE	TIME	TOTAL N	UREA + NH ₃ N	UREA + NH ₃ N
			gram	per cent
February 6	12.00-4.10	0.723	0.688	95
March 22	12.00-4.10	0.747	0.641	86
March 28	12.00-4.10	0.672(calc.)	0.616	92

If one now considers the variations in nitrogen elimination during the same hours when no Liebig's extract was given, these are found to be as much as 0.038 gram per hour as appears from the following table:

DATE	N PER HOUR	DATE	N PER HOUR
December 2	0.133	January 30	0.130
January 22	0.124	February 5	0.094
January 26	0.095	April 22	0.132

It appears from the above that the value of the nineteenth to the twenty-second hours after food ingestion is usually about 0.132 gram of nitrogen as found on April 22. Assuming a value

of 0.132 per hour, then 0.549 gram would be eliminated during four hours and ten minutes.

Considering now the elimination of nitrogen during the same interval of time after meat extract containing 0.156 gram of nitrogen was administered, the following differences are found:

Date	Total N	Extra N
February 6.....	0.723	$0.723 - 0.549 = 0.174$
March 22.....	0.747	$0.747 - 0.549 = 0.198$
March 28.....	0.672	$0.672 - 0.549 = 0.123$

It is, therefore, evident that the nitrogen ingested in the meat extract is essentially eliminated during the first four hours after its administration.

To estimate the protein metabolism of February 6, when meat extract was given, one might assume (1) the value of the hourly nitrogen elimination of the day previous ($= 0.094$), or (2) one might deduct from the nitrogen in the urine of the period the nitrogen content of the Liebig extract given ($0.723 - 0.156 = 0.567$) which gives an hourly excretion of 0.136 gram, a more probable value. A similar calculation for the urine of March 22 shows an hourly value of 0.142 gram of nitrogen.

The metabolism of February 6 has been calculated on the basis of both methods of estimating the protein nitrogen elimination with the following results:

Influence of small variations in protein metabolism upon the calculation of results.

TIME	N IN URINE	NON- PROTEIN R. Q.	CALORIES	N IN URINE	NON- PROTEIN R. Q.	CALORIES
1.00-2.00	0.094	0.88	15.15	0.136	0.88	15.06
2.00-3.00	0.094	0.82	16.48	0.136	0.81	16.37
3.00-4.00	0.094	0.73	17.71	0.136	0.72	17.62
			49.34			49.05

This table shows that however crude the method of analysis may appear to the critical, the general result is practically uninfluenced by a possible error of 0.042 gram of estimated protein nitrogen elimination per hour.

B. 25 grams of glycoll.

Since both carbon atoms contained in glycoll are convertible into dextrose, it may be calculated that 25 grams of glycoll produce 20 grams of dextrose. In paper three it was shown that 20 grams of dextrose containing 75 calories caused, during the second hour only, an increase of 2.5 calories in heat production—a specific dynamic action of 3.3. The question was, would glycoll behave in the same fashion?

The experiments were continued from the beginning of the second to the end of the ninth hour after the ingestion of the material, in two different series of four hours each and each series was done twice, making experiments which are essentially well agreeing duplicates (see table I). The important results appear in the following:

Experiments 44 and 48.

25 grams of glycoll (4.66 grams N and 46.15 calories) in 150 cc. of water plus 2 grams of Liebig's extract, at noon.

Values in calories.

TIME	PROTEIN	GLYCOLL	NON-PROTEIN	TOTAL CALCULATED	TOTAL FOUND
1.00-2.00	3.02	0.73	17.37	21.12	21.96
2.00-3.00	3.02	0.99	18.85	22.86	21.31
3.00-4.00	3.02	1.43	20.30	24.75	21.23
4.00-5.00	3.02	2.93	16.75	22.70	21.16
5.00-6.00	3.02	3.60	11.81	18.43	19.24
6.00-7.00	3.02	3.60	13.71	20.33	20.17
7.00-8.00	3.02	3.60	12.19	18.81	18.33
8.00-9.00	3.02	3.60	10.48	17.10	17.53
Total.....	24.16	20.48	121.46	166.10	160.93

The difference between calories calculated and calories found is 5.2 of which 3.6 falls in the single hour between three and four o'clock. The result of the glycoll ingestion was a decided rise in metabolism, which continued over a period of eight hours. During the ninth hour the metabolism fell nearly to the basal value, which has been determined as 16.2 calories per hour. For the eight hours at this level the basal metabolism would have been 129.6 calories. Taking the lower figure, "calories found" in the

above experiment, the heat production after giving 25 grams of glycocoll amounted to 160.9 calories for a period of eight hours, an increase of 31.3 calories above the basal value. Since the ingested glycocoll yields 46.15 calories in metabolism, it may be calculated that *the specific dynamic action of glycocoll is 70*. Using a calculation (see below) which indicates that 8 per cent of the absorbed glycocoll is eliminated unchanged in the urine, the specific dynamic action may be estimated to be 74. This is calculated after the manner of Rubner. If, however, one considers the very low level of glycocoll metabolism during the second and third hours as measured by the extra urea plus ammonia excretion, it is apparent that, although no considerable quantity of glycocoll is destroyed, yet the heat production is about at its maximum during these hours. There is a rise of 5 calories above the basal metabolism, during the second hour, although the calories from glycocoll metabolism are indicated as being only 0.73 (or only 3.15 calories if the estimated glycocoll metabolism of the next two hours be included). In the interpretation of these results, valued information is to be obtained from the work of Folin and Denis.⁸ These authors find that one hour after the administration of about 2 grams of glycocoll to anesthetized cats, the "non-protein nitrogen" (which must contain absorbed glycocoll) has increased in the blood and in the tissues, but there has been no increase in the quantity of urea nitrogen in either locality. The urea formation begins about an hour after the ingestion of the material. From one experiment (in which the kidney function was undisturbed) the following details are reproduced.

Glycocoll given, 1.867 grams; absorbed, 0.917 grams.

Values, milligrams in 100 grams substance.

TIME	CAROTID BLOOD		MUSCLE	
	UREA N	NON-PROTEIN N	UREA N	NON-PROTEIN N
At start.....	34	60	42	248
After 2 hours.....	44	100		
After 3 hours.....	50	101		
After 4 hours.....	60*	124*	54	304

* Iliac artery.

⁸ Folin and Denis: *This Journal*, xii, p. 141, 1912, and their previous papers.

It appears from this that the absorbed amino-acids circulate in the blood, and are retained in the muscle tissue, while after one hour the urea rises in the blood in response to the increased production of urea in the tissues. These results are interpretative of the nitrogen figures representing the glycocoll metabolism in the dog as shown above, and indicate that they give an approximate idea of the actual happenings.

Since there is a great rise in metabolism during the second hour after glycocoll ingestion and this is not accompanied by a large metabolism of absorbed glycocoll, it is evident that *glycocoll acts as a stimulus to increase the oxidative processes in the organism*. Furthermore, since the high metabolism is found during the second hour when the urea production from glycocoll is still low, it appears that *the increased metabolism is due to the direct stimulus of glycocoll itself and not to any process concerned with intermediary metabolism such as the process of deaminization*.

Comparison to show the similarity between the metabolism after the ingestion of 20 grams of dextrose which exerts a specific dynamic action of 3.2 with that produced by its isoglucosic equivalent, 25 grams of glycocoll, which exerts a specific dynamic influence of 70, breaks down completely. Most dogs drink a solution of dextrose of their own free will. They drink a solution of glycocoll the first time it is offered to them, but not readily after that. Glycocoll solutions are often vomited and some dogs never retain them. Both materials have a sweet taste, and yet their action on the stomach wall, as well as upon the cells of the organism in general, is distinctly different. It may be suggested that the action of glycocoll is merely through the sensory stimulation of the mucous membrane of the stomach and intestines, but the continuation of the increased metabolism for seven or eight hours after the ingestion of the material and long after the sensation of nausea has passed away, disproves this. Furthermore, urea solutions which produce nausea and often vomiting, have no influence whatever upon metabolism as shown in paper three. In the experiments here described, the dog betrayed no signs of nausea but in the two instances remained in the calorimeter without moving during the first hour of the experiment.

In the work of Ringer and Lusk⁹ it was shown that the nitrogen and sugar which arise from the ingestion of 20 grams of glycocholate by a phlorhizinized dog are usually completely eliminated in twelve hours. In the present experiment it is found that the maximal action on metabolism takes place during the second to fifth hours, during which the mass action of the entering amino-acids may be greatest. With the completion of the absorption and the destruction of the amino-acids as such, this mass action upon the cells diminishes with an accompanying diminution in the intensity of metabolism.

It will be noted that the behavior of glycocholate is entirely similar to the behavior of meat, as has been set forth in paper two of this series. Glycocholate, the simplest of the amino-acids, has the most profound effect of all those which have been studied. How important the effect of glycocholate upon the general metabolism is, cannot, with certainty, be stated until it is known whether the large amount excreted after the administration of benzoate of soda¹⁰ corresponds to the amount produced normally.

The urinary analyses. It has been admitted that the calculations of the hourly nitrogen values after administration of Liebig's extract could not be exactly calculated and the calculations become even more complex when amino-acids are given with Liebig's extract. The following general procedure has been used. (1) The values of total N and urea plus ammonia N were determined in hourly periods after giving Liebig's extract. (2) Total N and urea plus ammonia N were determined in hourly periods for six hours after giving 25 grams of glycocholate plus Liebig's extract. (3) These last values have been assumed as correct during the period when the dog was in the calorimeter. (4) The protein metabolism of the time has been taken as an assumed value of the probable nitrogen elimination had no Liebig's extract or amino-acid been ingested. (5) The difference between the urea plus ammonia nitrogen when Liebig's extract was given alone and when it was given with glycocholate, forms the basis of the determination of the amount of glycocholate metabolized. (6) The increase in total nitrogen due to the addition of glycocholate to Liebig's extract, minus

⁹ Ringer and Lusk: *loc. cit.*

¹⁰ Magnus-Levy: *Munch. med. Wochenschr.*, lii, p. 2168, 1905; Ringer: *this Journal*, x, p. 327, 1911.

the nitrogen of metabolized glycocoll, represents the amount of glycocoll which was eliminated unchanged.

The above presents in general outline the method of all the determinations made with various amino-acids in this paper. Exceptions to this procedure will be mentioned when they occur.

The following table gives the results obtained after ingesting glycocoll.

Urinary analyses after glycocoll ingestion.

TIME	MARCH 28		MARCH 25		DIFFERENCE BETWEEN UREA PLUS NH ₃ N = GLYCOCOLL N ME- TABOLIZED	DIFFERENCE BETWEEN TOTAL N MINUS GLY- COCOLL N METABA- LIZED = GLYCOCOLL N UNMETABOLIZED
	2 GRAMS LIEBIG'S EXTRACT IN 150 CC. WATER		25 GRAMS GLYCOCOLL PLUS 2 GRAMS LIEBIG'S EXTRACT IN 150 CC. WATER			
	Total N	Urea + NH ₃ N	Total N	Urea + NH ₃ N		
12.00-1.00	0.154	0.152	0.242	0.198	0.056	0.034
1.00-2.00	0.169	0.162	0.361	0.236	0.074	0.118
2.00-3.00	0.176	0.156	0.315	0.256	0.100	0.039
3.00-4.00	0.150	0.126	0.381	0.276	0.150	0.081
4.00-5.00	0.140	0.122	0.463	0.418	0.296	0.027
5.00-6.00	0.114	0.104	0.416	(0.348)		
Total.....	0.903	0.822 = 91 %	2.178	1.732 = 78 %	0.676	0.299

On the calorimeter days, March 29 (Exp. 41) and April 4 (Exp. 44), the actual elimination of nitrogen and the elimination assumed on the basis of the analyses of March 25 are given below:

DATE	TIME	TOTAL N	UREA + NH_3 N	PER CENT
March 29, Exp. 41.....	12.00-5.10	1.583	1.355	85
March 25 (est.).....	12.00-5.10	1.839	1.454	
April 4, Exp. 44.....	12.00-5.08	1.825	1.578	86
March 25 (est.).....	12.00-5.08	1.823	1.403	

The results of the actual and calculated values of April 4 show little differences. Greater differences are shown on March 29, the nitrogen elimination being 14 per cent less than on March 25. The assumed values were, however, adopted. This was justified because the heat production in both experiments, 41 and 44, was essentially the same. More perfect results than these could have been

obtained if the dog had been provided with a catheter and an apparatus for the automatic washing of the bladder at the end of each hourly period while in the calorimeter. Although this could have been arranged, yet it was deemed undesirable on account of the necessity of keeping the dog in complete repose.

On March 28, the day on which Liebig's extract alone was given, the total nitrogen was 0.114 and the urea plus ammonia N was 0.104 between five and six o'clock. The value 0.114 N has been assumed as representing the fasting protein metabolism in the following experiments after glycocoll ingestion at noon.

On April 13 and 23, the dog was placed in the calorimeter and his metabolism determined between five and nine o'clock. The urine of the whole period was analyzed and was partitioned equally through the several hours. The results were:

DATE	EXP. NO.	TIME	TOTAL N	UREA+NH ₃ N	PER CENT
April 13	48	4.00-9.10	2.425	2.297	95
		Per hour	0.469	0.445	
April 23	52	4.00-9.10	2.642	2.478	94
		Per hour	0.511	0.479	

Since the urea plus ammonia elimination when no glycocoll is ingested is 0.104 gram per hour (see above) the nitrogen elimination representing glycocoll metabolized would be in Experiment 48, 0.341 gram per hour, and in Experiment 52, 0.375 gram per hour. The high percentage of urea plus ammonia nitrogen during this period of four hours shows that practically no glycocoll was eliminated in the urine as such; for, calculated on the basis before described, only 0.014 and 0.022 gram of glycocoll nitrogen per hour could be estimated in the urine. The values of 0.469 and 0.511 gram of total N metabolized per hour between four and nine o'clock agree with the values found on March 28 of 0.463 and 0.461 for the two hours between four and six. It is assumed that the urea elimination during the following three hours did not essentially vary from this.

The method of determining the nitrogen elimination of the dog within the calorimeter as described above was also used in the following experiments with other amino-acids, and need not again be described.

C. 20 grams of i-alanine.

Since 20 grams of *i*-alanine are convertible in the organism into 20 grams of dextrose, that quantity was given in Liebig's extract of beef and the metabolism determined in hourly periods from the second to fifth hour to discover whether its behavior was different from that of dextrose.

The results were as follows:

Experiment 43.

20 grams of alanine (3.14 grams N and 60.7 calories) in 150 cc. of water plus 1 grams of Liebig's extract at noon.

Values in calories.

TIME	PROTEIN	ALANINE	NON- PROTEIN	TOTAL CAL- CULATED	TOTAL FOUND
1.00-2.00	3.50	2.28	13.96	19.74	19.45
2.00-3.00	3.50	2.80	12.17	18.47	19.19
3.00-4.00	3.50	5.15	11.29	19.94	19.39
4.00-5.00	3.50	5.23		(19.26)	19.26
Total.....	14.00	15.46		77.41	77.29

The basal metabolism of the dog was 16.2 calories per hour or 64.8 calories during four hours. Hence the ingestion of 20 grams of alanine caused an increase of 12.61 calories during four hours, which shows a specific dynamic action of 21 during this short period. Folin and Denis (*loc. cit.*) have shown that there is no increase in urea in the blood during the first hour after alanine ingestion, but that the quantity of alanine increases in the blood. During the second hour urea formation commences.

It will be noticed that, although the quantity of alanine metabolized increased hour by hour, the heat production reached its maximum during the second hour.

The behavior of alanine appears to be similar to that of glyco-coll, but alanine acts as a less powerful stimulus. Glycocoll caused a rise of 5 calories during the first hour, whereas alanine caused one of 3.5 calories.

The urinary analyses. The urinary analyses were computed as in the subjoined table:

Urinary analyses after alanine ingestion.

TIME	MARCH 28		APRIL 3		DIFFERENCE BETWEEN UREA PLUS NH_3 N = ALANINE N ME- TABOLIZED	DIFFERENCE BETWEEN TOTAL N MINUS ALA- NINE N METABO- LIZED = ALANINE NOT METABOLIZED
	2 GRAMS LIEBIG'S EXTRACT IN 150 CC. WATER		20 GRAMS ALANINE PLUS 2 GRAMS LIEBIG'S EXTRACT IN 150 CC. WATER			
	Total N	Urea + NH_3 N	Total N	Urea + NH_3 N		
12.00-1.00	0.154	0.152	0.197	0.136	0	0.043
1.00-2.00	0.169	0.162	0.431	0.280	0.118	0.144
2.00-3.00	0.176	0.156	0.461	0.301	0.145	0.140
3.00-4.00	0.150	0.126	0.562	0.393	0.267	0.145
4.00-5.00	0.140	0.122	0.515	0.393	0.271	0.104
Total.....	0.789	0.718 = 91 %	2.166	1.503 = 70 %	0.801	0.576

The above figures show, in confirmation of Folin, that there is no urea production during the first hour after giving alanine. There appears to be a large excretion of unoxidized alanine amounting in five hours to 18 per cent of the material ingested. The metabolism of alanine as measured by the increase in urea plus ammonia N rose hour by hour.

On the calorimeter day, April 1, Experiment 43, the hourly protein metabolism was assumed to be represented by 0.132 grams N, an amount which was eliminated on April 22, when no food was given at noon; the amino-acid metabolism was estimated from the computation shown above, which the following comparison justifies.

DATE	EXPERI- MENT	TIME	TOTAL N	UREA + NH_3 N	PER CENT
April 1	43	12.00-5.10	2.19	1.53	70
April 3	(est.)	12.00-5.10	2.25	1.56	70

D. 20 grams of i-glutamic acid.

It has been stated that glutamic acid yields three of its carbon atoms as glucose in the diabetic organism, whereas two are oxidized. Here appeared a chemical complex which might serve to

settle the theory of the specific dynamic action of protein. The following serves to illustrate the results of its ingestion:

Experiment 49.

20 grams of glutamic acid (1.90 grams N and 62.9 calories) in 150 cc. of water plus 2½ grams of Liebig's extract at noon.

Values in calories.

TIME	PROTEIN	GLUTAMIC ACID	NON-PROTEIN	TOTAL CALCULATED	TOTAL FOUND
1.00-2.00	3.50	1.62	10.09	15.21	15.49
2.00-3.00	3.50	4.96	7.70	16.16	15.26
3.00-4.00	3.50	5.16	8.25	16.91	15.99
4.00-5.00	3.50	6.68	6.82	17.00	16.59
Total.....	14.00	18.42	32.86	65.28	63.33

The average between the calories "found" and "calculated" is 64.3 which may be compared with a basal metabolism of 64.8 calories for the period.

Glutamic acid exerts no "specific dynamic action" whatsoever. It is an amino-acid which is an important constituent of most protein and yet its ingestion does not increase the heat production, although it is freely absorbed and deaminized. Therefore, *the process of deamination and urea production are not concerned with the heat increase accompanying specific dynamic action.* One may also draw the conclusion that *glutamic acid cannot yield glycocoll as an intermediary product.*

If the behavior of glutamic acid is on account of its chemical configuration, then one must assume that *aspartic acid* behaves in a similar manner in metabolism, although no experiments have been made with this substance.

The urinary analyses. As in the last experiment the protein metabolized was assumed to be represented by a urinary elimination of 0.132 gram N per hour. The remaining factors were computed as follows:

Urinary analysis after glycocoll ingestion.

TIME	MARCH 28		APRIL 16		DIFFERENCE BETWEEN UREA PLUS NH ₃ N = GLUTAMIC ACID METABOLIZED	DIFFERENCE BETWEEN TOTAL N MINUS GLU- TAMIC ACID N = GLUTAMIC ACID UN- METABOLIZED
	2 GRAMS LIEBIG'S EXTRACT IN 150 CC. WATER		20 GRAMS GLUTAMIC ACID PLUS 2 GRAMS LIEBIG'S EXTRACT IN 150 CC. WATER			
	Total N	Urea + NH ₃ N	Total N	Urea + NH ₃ N		
12.00-1.00	0.154	0.152	0.246	0.208	0.056	0.036
1.00-2.00	0.169	0.162	0.289	0.211	0.049	0.073
2.00-3.00	0.176	0.156	0.406	0.306	0.150	0.080
3.00-4.00	0.150	0.126	0.337	0.282	0.156	0.031
4.00-5.00	0.140	0.122	0.368	0.324	0.202	0.026
Total.....	0.789	0.718 = 91 %	1.646	1.331 = 81 %	0.613	0.246

Comparison of the analysis of the urine of April 17, the day on which the dog received glutamic acid and was placed in the calorimeter with that of April 16 with the same diet outside the calorimeter, showed the following relations:

DATE	EXPERIMENT	TIME	TOTAL N	UREA + NH ₃ N
April 17	49	12.00-5.10	1.509	1.397
April 16	(est.)	12.00-5.10	1.707	1.387

E. 20 grams of i-leucine.

The following results were obtained after giving leucine:

Experiment 50.

20 grams of leucine (2.14 grams N and 118.9 calories) in 150 cc. of water plus 2 grams of Liebig's extract at noon.

Values in calories.

TIME	PROTEIN	LEUCINE	NON- PROTEIN	TOTAL CAL- CULATED	TOTAL FOUND
1.00-2.00	3.50	7.64	6.45	17.59	17.06
2.00-3.00	3.50	6.35	6.88	16.73	17.40
3.00-4.00	3.50	6.74	7.46	17.70	17.68
4.00-5.00	3.50	7.57	8.27	19.34	18.36
Total.....	14.00	28.30	29.06	71.36	70.50

It appears from the above, that in spite of its insolubility, a noticeable quantity of leucine was absorbed and metabolized during the first five hours after its ingestion. Its action in raising the heat production was not great, the basal metabolism of 64.8 calories being increased by only 6.1 calories or about 10 per cent.

The urinary analyses. The value 0.132 gram N was again assumed to represent the nitrogen of the true protein metabolism of the time. The other figures were determined as follows:

Urinary analyses after leucine ingestion.

TIME	MARCH 28		APRIL 18		DIFFERENCE BETWEEN UREA PLUS NH ₃ N = LEUCINE METAB- OLIZED	DIFFERENCE BETWEEN TOTAL N MINUS (a)- CINE N = LEUCINE N NOT METABOLIZED
	2 GRAMS LIEBIG'S EXTRACT IN 150 CC. WATER		20 GRAMS LEUCINE PLUS 2 GRAMS LIEBIG'S EXTRACT IN 150 CC. WATER			
	Total N	Urea + NH ₃ N	Total N	Urea + NH ₃ N		
12.00-1.00	0.154	0.152	0.149	0.133	0	0
1.00-2.00	0.169	0.162	0.329	0.299	0.137	0.225
2.00-3.00	0.176	0.156	0.298	0.270	0.114	0.008
3.00-4.00	0.150	0.126	0.270	0.247	0.121	0
4.00-5.00	0.140	0.122	0.278	0.258	0.136	0.002
Total.....	0.789	0.718 = 91 %	1.324	1.207 = 91 %	0.508	0.035

The above shows no elimination of leucine nitrogen during the first hour but an elimination of a quarter of the nitrogen ingested during the following four hours. Very little or no leucine could have been excreted as such in the urine.

On April 19, the dog was given 20 grams of leucine with Liebig's extract and was then placed in the calorimeter. The urine of this day in comparison with that of April 18 showed the following:

DATE	EXPERI- MENT	TIME	TOTAL N	UREA + NH_3 N	PER CENT
April 19	50	12.00-5.04	1.481	1.244	84
April 18	(est.)	12.00-5.04	1.324	1.207	91

F. 20 grams of tyrosine.

The results of giving 20 grams of tyrosine are presented in the following:

Experiment 46.

20 grams of tyrosine (1.55 grams N and 109.9 calories) in 150 cc. of water plus 2 grams of Liebig's extract at noon.

Values in calories.

TIME	PROTEIN	TYROSINE	NON-PROTEIN	TOTAL CAL-CULATED	TOTAL FOUND
1.00-2.00	3.50		14.43	17.93	16.56
2.00-3.00	3.50		14.12	17.62	17.20
3.00-4.00	3.50		16.39	19.89	18.56
4.00-5.00	3.50		14.68	18.18	17.14
Total	14.00		59.62	73.62	69.46

It appears from this table that the metabolism was slightly increased after giving 20 grams of tyrosine. If the calories "found" and "calculated" be averaged, the result is 71.5 as against a basal value of 64.8 calories, a gain of 7.7 calories, or 10 per cent for the period. The result is, therefore, similar to that obtained with leucine, except that in the case of leucine, there was a largely increased elimination of urea plus ammonia nitrogen which is not evident with tyrosine. It is possible that tyrosine, even though only small quantities are absorbed, will act as a stimulus upon the cells (see Summary).

The urinary analyses. After giving tyrosine, there was no increase in the nitrogen elimination during five hours which could not be ascribed to experimental error. The following compares the results found in the experiment after giving 20 grams of tyrosine in Liebig's extract with the figures for Liebig's extract alone.

DATE	EXPERIMENT	TIME	TOTAL N	UREA + NH ₃ N	PER CENT
April 10	46	12.00-5.08	0.888	0.786	89
March 28	(est.)	12.00-5.08	0.808	0.733	91

G. 5.5 grams each of glycocoll, alanine, glutamic acid, leucine and tyrosine.

It was deemed of interest to determine the action of a mixture of amino-acids which might be compared with 100 grams of meat. To this end a mixture of the five amino-acids already described, consisting of 5.5 grams of each substance and being 27.5 grams in all, was prepared. The values for the calculation of the results were computed from the table given on p. 158 and were as follows:

Mixed amino-acids contain 12.47 per cent N.

Average heat value per gram = 3.894 calories.

Average heat value per gram N = 31.23 calories.

1 gram mixed acids yields 1.474 grams CO₂ for respiration.

1 gram mixed acids requires 1.242 grams O₂ for oxidation.

1 gram N of mixed acids yields 11.82 grams CO₂ for respiration.

1 gram N of mixed acids requires 9.96 grams O₂ for oxidation.

The results obtained are given below.

Experiment 45.

5.5 grams each of glycocoll, alanine, glutamic acid, leucine and tyrosine (3.46 grams N and 107.1 calories) in 150 cc. of water plus 2 grams of Liebig's extract at noon.

Values in calories.

TIME	PROTEIN	AMINO-ACIDS	NON-PROTEIN	TOTAL CALCULATED	TOTAL FOUND
1.00-2.00	3.50*	8.06*	9.81*	21.37	22.27
2.00-3.00				21.37	21.77
3.00-4.00				21.37	19.25
4.00-5.00				21.37	20.09
Total.....				85.48	83.38

* Values per hour throughout.

The average of these results shows a metabolism amounting to 84.4 calories, an increase of 19.6 above the basal value. No such height of metabolism has been found except in the case of the ingestion of 25 grams of glycocoll. If the "calories found" in the two experiments be compared, the results are seen to be in close agreement as appears from the following:

TIME	25 GRAMS GLYCOCOLL	27.5 GRAMS MIXED AMINO-ACIDS
	Calories found	Calories found
1.00-2.00	21.96	22.27
2.00-3.00	21.31	21.77
3.00-4.00	21.23	19.25
4.00-5.00	21.16	20.09
Total.....	85.66	83.38

Since the mixture of 27.5 grams raises the metabolism to the same height as 25 grams of its most powerful component, and not to the height of the average of all, it appears probable that the amino-acids of lower specific dynamic action each acts specifically, each contributing its total to the general result. The total result would then be dependent upon the mass action of the individual units. It seems improbable that the whole result could have been determined by the action of 5.5 grams of glycocoll alone although this cannot now be stated with certainty. It must be recalled in this connection that glutamic acid is without influence, so the result was really produced by 22 grams containing 2.94 grams of N and made up of equal portions of glycocoll, alanine, leucine and tyrosine, of which only the first two act powerfully.

The urinary analyses. The urine was not separately obtained in hourly periods, but only the urine of the calorimeter period was collected and the calculations are based upon this whole period. The nitrogen found, less the nitrogen obtained when Liebig's extract was given alone, gives the following results:

DATE	EXP.	TIME	TOTAL N	UREA+NH ₃ N	PER CENT
April 8	45	12.00-5.12	2.039	1.804	88
March 28	(est.)	12.00-5.12	0.792	0.720	91
Total.....			1.247	1.084	87

It appears that 87 per cent of the increase in nitrogen excretion due to the ingested amino-acids, was in the form of urea or ammonia. Since practically none of this increase ever appears in the

urine during the first hour after the ingestion of amino-acids, the urea plus ammonia nitrogen was divided by 4.2 hours to determine the average hourly metabolism of amino-acid, which gave the value of 0.258 gram of N.

H. 100 grams of meat.

In paper two it was shown that ingestion of 1000 grams of meat by the same dog used in these experiments, caused the metabolism to rise from 16.2 to 30 calories per hour. The influence of 100 grams of meat from beef heart is indicated below:

Experiment 47.

100 grams of meat (3.0 grams N and 78 protein calories).

Values in calories.

TIME	PROTEIN	NON-PROTEIN	TOTAL CALCULATED	TOTAL FOUND
1.00-2.00	9.09	10.37	19.46	20.02
2.00-3.00	10.52	9.68	20.20	19.81
3.00-4.00	8.85	11.94	20.79	18.80
Total.	28.46	31.99	60.45	58.63

The average metabolism during three hours may be estimated at 59.55 calories, an increase of 11 above the basal metabolism. Assuming for the sake of comparison, that the metabolism during a fourth hour is maintained at the average height of 19.8 calories hourly, as one may on account of the nitrogen in the urine during this hour, the total metabolism may be estimated at 79.3 calories during four hours, and the results may be compared with those obtained after giving 27.5 grams of mixed amino-acids, as follows:

	100 grams meat (3 grams N and 78 protein calories)	27.5 grams mixed amino-acids (3.48 grams N and 107 calories).
Calories in four hours.....	79.3	84.4
Increase above basal value.....	14.5	19.6

Since, as has been said, glutamic acid exerts no heat increasing function, it may be concluded that 22 grams made up of glycocoll,

alanine, tyrosine and leucine in equal amounts and containing together 2.94 grams of N and 90 calories, exerts a more profound influence upon metabolism than does 100 grams of meat containing 3 grams of N and 78 protein calories, during an experimental period which includes the second to fourth or fifth hours after their ingestion. The higher metabolism after giving the mixture of amino-acids may, in part, be due to the greater rapidity of their absorption and in part to the particular constituents of the mixture.

The urinary analysis. On April 9, 100 grams of beef-heart were given at noon and the hourly nitrogen excretion showed the following results:

TIME	TOTAL N grams
12.00-1.00.....	0.208
1.00-2.00.....	0.343
2.00-3.00.....	0.397
3.00-4.00.....	0.334
4.00-5.00.....	0.407
Total.....	1.689

On April 11, when the dog was in the calorimeter, 1.466 grams of N were eliminated between the hours of twelve and five o'clock.

IV. GENERAL STATISTICS.

In the above research, various amino-acids have been given under such conditions that their action during the second to fifth hours after ingestion are strictly comparable. The following table gives a summary of the results.

Table indicating the specific dynamic action of various amino-acids, separately, together and compared with meat, during an interval of four hours.

SUBSTANCE	CALORIES OF INGESTA	CALORIES OF METABOLISM	INCREASE IN CALORIES OF METABOLISM	100 CALORIES OF INGESTA CAUSES INCREASE IN METABOLISM IN CALORIES
No food.....	0	64.8		
25 grams glycocoll (4.66 grams N).....	46.15	85.7	20.9	45
20 grams alanine (3.14 grams N).....	60.7	77.4	12.6	21
20 grams glutamic acid (1.90 grams N).....	62.9	64.3	0.0	0
20 grams leucine (2.14 grams N).....	118.9	70.9	6.1	5
20 grams tyrosine (1.55 grams N).....	109.9	71.5	7.7	7
5.5 grams of each of above (3.46 grams N)	107.1	84.4	19.6	19
100 grams meat (3 grams N).....	78.0	79.3*	14.5	19

* Last hour estimated.

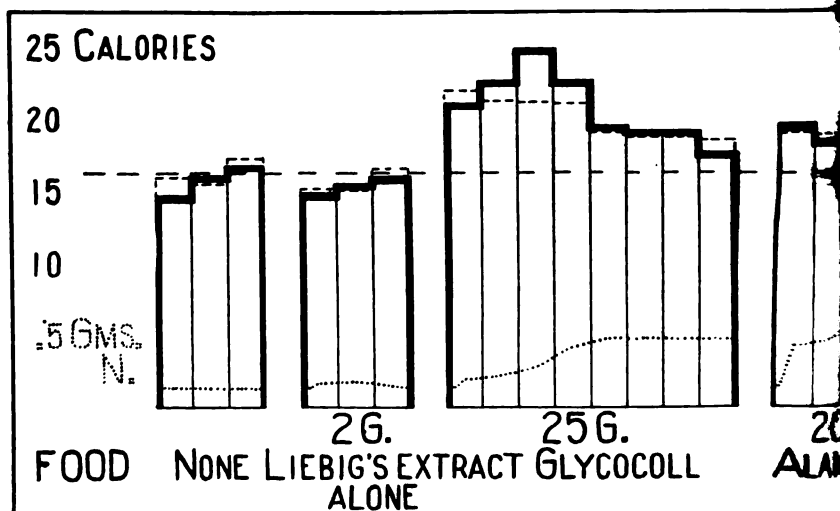
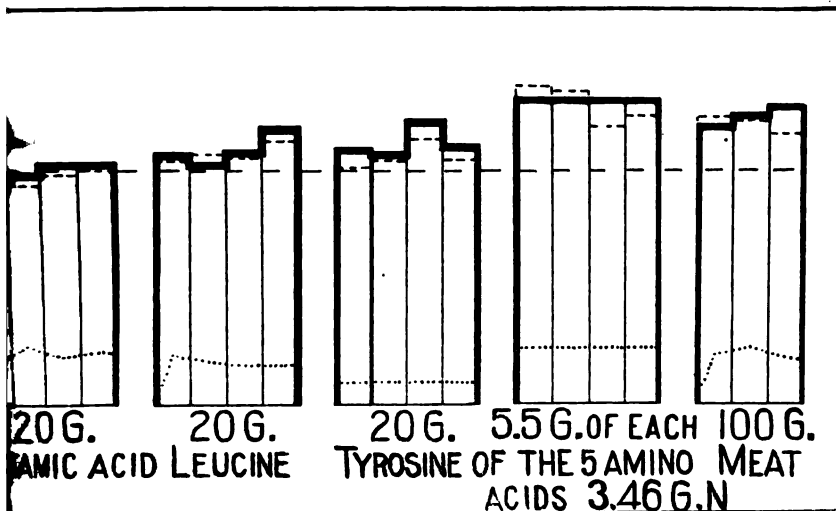


CHART 1.—DOG II. HOURLY METABOLISM BEGINNING TH
Heavy line—Calories calculated. Broken line—Calories found. Dotted line—

The preceding table shows that the simplest amino-acid, glyco- coll, has the most powerful action and that alanine also has a pronounced effect. Leucine and tyrosine, per gram of substance, exert about half the effect of alanine, while glutamic acid, with its two carboxyl groups, is without influence. The increase in the number of calories after giving 27.5 grams of the mixed acids is almost as great as when 25 grams of glycocoll were given. The accompanying chart shows many of the essential features of the work.

The metabolism of the amino-acids was at a higher level when they were mixed together than when they were given alone, as is indicated by the following table:

	Urinary N from amino- acids, metabolised dur- ing five hours.
	grams
Glycocoll.....	0.676
Alanine.....	0.801
Glutamic acid.....	0.613
Leucine.....	0.508
Tyrosine.....	0.0
Mixed amino-acids.....	1.084



UR AFTER GIVING AMINO-ACIDS WITH LIEBIG'S EXTRACT.
protein plus amino-acid metabolized.

Another point of general interest is that the ingestion of Liebig's extract alone or with glutamic acid did not cause a rise in body temperature, whereas those amino-acids which strongly increased metabolism also caused the body temperature to rise.

V. SUMMARY.

Near the end of paper two, the following statement was made with regard to metabolism after meat ingestion: "The intensity of the metabolism must be due either to the intensity of the intermediary processes of protein metabolism itself, or more probably to the degree of mass action of amino-acids acting as stimuli upon the cellular protoplasm."

The first alternative accords with the opinions of Rubner, the second has been reached as the result of the present series of experiments. Rubner's theory explained the facts revealed by his experiments. It appeared that in the breaking up of the variety of amino-acids constituting protein, free heat might be liberated which could in no wise be used as power for the machinery of the cells, whereas certain products of protein metabolism such as sugar could be so utilized.

Although it is admitted that after giving meat or amino-acids to dogs, the metabolism has never been increased during a long period so that the increase could not have been derived wholly from the metabolism of the ingesta, yet there are many reasons which lead to the belief that the increased heat production is not entirely or even mainly due to intermediary processes taking place at the expense of ingested protein.

The reasons for believing that incoming amino-acids, in proportion to their mass action, stimulate the cell substance to higher oxidation are as follows:

1. After giving 1200 grams of meat, the metabolism during the second hour rose almost to its maximum height, although the urinary nitrogen reached only a third of its maximum (see paper two). The respiratory quotient for this period may be found to be over 0.90 and, therefore, it appears that carbohydrate and not additional protein is oxidized during this period. Calculated on the basis of the increase in urinary nitrogen, the specific dynamic action was 90. After giving 700 grams of meat to the same dog, the specific dynamic action for the same second hour was 139,

and for the third hour it was 106. In other words, the increase in heat production was greater during these hours than could have been derived from the extra protein metabolism of the time.

2. After the ingestion of amino-acids, the most powerful effect was produced with glycocoll. After giving 25 grams of glycocoll, there was an increased heat production during a period of seven hours of experimentation which was equal to 70 per cent of the energy in the glycocoll ingested. However, the maximum increase in metabolism was reached as early as the second hour. To throw further light upon what happens, one must recall again the recent experiments of Folin and Denis¹¹ who show that, during the first hour after glycocoll ingestion, glycocoll is absorbed by the blood and is retained by the blood and muscle, but there is no urea formation until the second hour. During the second and immediately following hours, urea constantly increases in the blood and increases in the muscle. This accords with the fact that after giving glycocoll no increased amount of nitrogen was found in the urine of the first hour, although the urine of the second hour contained an increased quantity. During this second hour the metabolism was raised by 5 calories, while the increased urea output indicated a metabolism of glycocoll which was the equivalent of only 0.73 calorie. During three hours the metabolism was raised 15 calories, while the glycocoll metabolism could be estimated at only 3.15 calories, which was less than the increased heat production of the second hour alone. The process of deamination and urea formation has nothing to do with this increase in metabolism, for there is no increased heat production when glutamic acid is oxidized. It seems, therefore, that the increase in metabolism during the second hour after giving glycocoll must be due to a direct stimulus upon the cells.

The action of alanine is similar but less powerful than glycocoll. Leucine also shows some effect. Tyrosine may influence the metabolism causing a slight increase during a four-hour period without causing any increase in the nitrogen output in the urine. This action may be interpreted in the light of the work of Folin and Denis¹² who showed that, although there was no increase in the urea content of blood or muscle three hours after adminis-

¹¹ Folin and Denis: *loc. cit.*

¹² *Ibid.*

tration of tyrosine, yet by a colorimetric test they were able to unmistakably prove the presence of tyrosine, in both blood and muscle.

3. A mixture, made up of 5.5 grams of each of the five amino-acids, glycocoll, alanine, glutamic acid, leucine and tyrosine, showed a more rapid metabolism of the constituent amino-acids than when they were given alone, and the effect on metabolism was as pronounced as that seen after giving 25 grams of glycocoll. It was greater than that of 100 grams of meat which contained about the same quantity of nitrogen.

4. The influence of glycocoll and alanine are not due to the fact that they produce nausea, for urea solutions also produce nausea but have no effect on the metabolism.

The influence is not due to movements of the intestines, for Benedict and Emmes¹³ have shown that diarrhoea produced by saline cathartics does not influence metabolism. Nor is the influence of glycocoll due to excitation of the digestive glands, for Liebig's extract of beef, which is known to induce glandular activity, is without influence upon the general heat production.

5. Since glycocoll and alanine are completely convertible into dextrose in phlorhizin glycosuria, and since the production of sugar from protein has been shown to be a normal function (see paper two), it follows, from Rubner's hypothesis, that glycocoll and alanine should exert no more influence upon metabolism than would a corresponding quantity of glucose. However, it has been shown that these two amino-acids, which are completely convertible into sugar, cause a very great increase in heat production. They do not act like sugar, into which they are convertible, but they act in virtue of their own special configuration. They do not act through the metabolism products which they form, for these are practically without effect as heat-increasing factors, but they act as stimuli upon the protoplasm with which they come in contact.

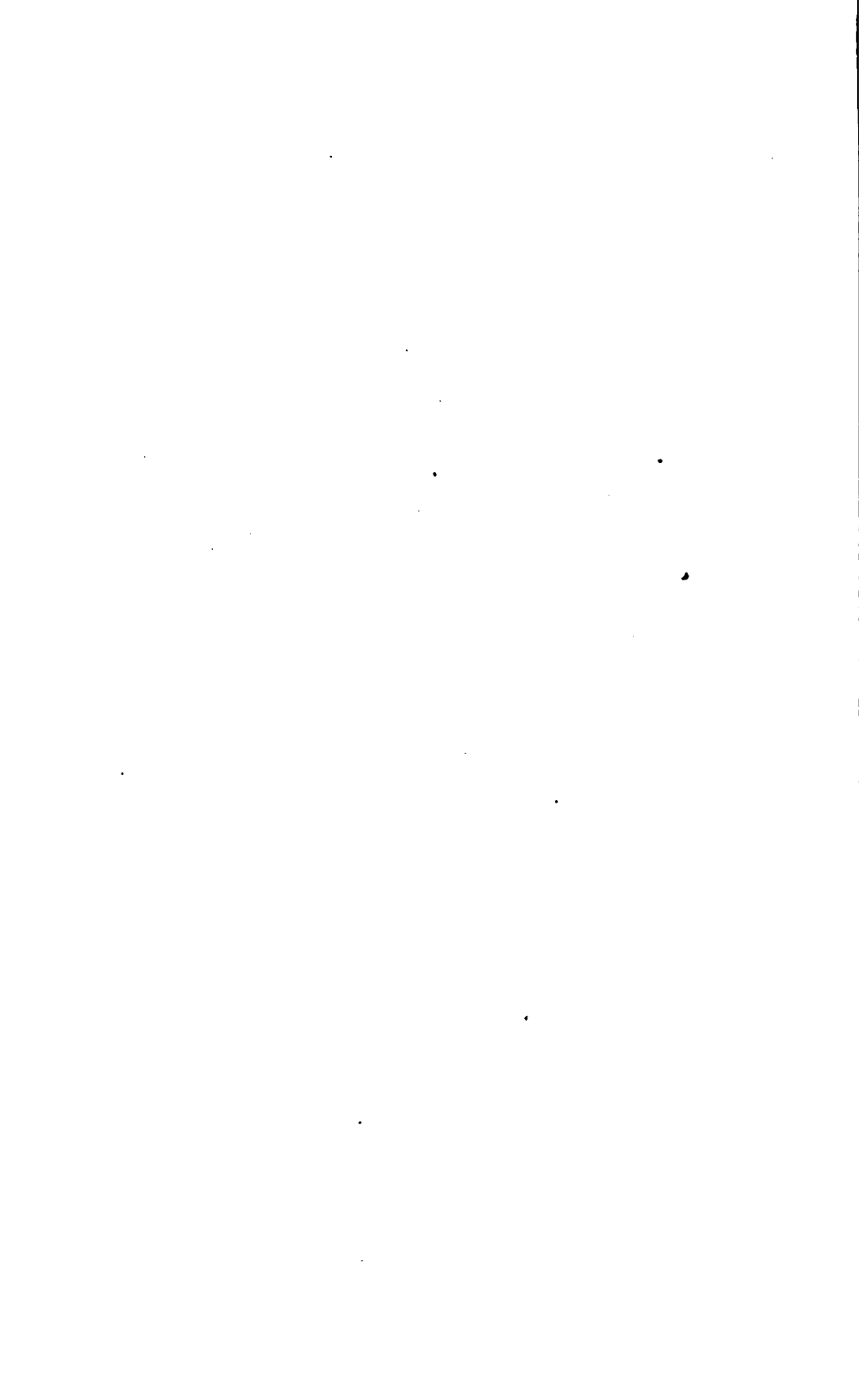
6. The work of Howland¹⁴ accomplished in this laboratory, has shown that an infant seven months old when given a modified milk administered in six equal portions during twenty-four hours and containing 3.58 grams of nitrogen in the day's diet produced during sleep 14.91 calories per hour. When 30 grams of casein

¹³ Benedict and Emmes: *Amer. Journ. of Physiol.*, xxx, p. 197, 1912.

¹⁴ Howland: *Zeitschr. f. physiol. Chem.*, lxxiv, p. 1, 1911.

were added to this diet and the nitrogen of the ingesta was 7.85 grams, the metabolism of the sleeping infant rose to 18.81 calories per hour, an increase of 3.9 calories or 26 per cent more than its former value. The casein added to the diet was the equivalent of 5.5 calories per hour. The urine of the period before the high protein diet contained 2.20 grams of N per day, and, during the day of the high protein diet, 4.46, an increase of 2.26 grams. This increase corresponds to 2.6 calories per hour. An increase in protein metabolism of 2.6 calories per hour brought about an increase of 3.9 calories in the metabolism of the sleeping infant. This is the only instance in which the metabolism may be seen to be permanently raised above the calorific value of the extra protein metabolism. The explanation may lie in the fact that the youthful protoplasm is more sensitive to stimuli than that of the adult. In Dog I, the basal metabolism was 759 calories per square meter of surface in twenty-four hours, in Dog II it was 784, in a dwarf nineteen years old it was 840, whereas in sleeping infants of three and seven months, it ranged between 1000 and 1100. These results could be obtained on account of comparable conditions of perfect rest.

As a final conclusion, it may be stated that *the increase in metabolism after the ingestion of meat is due to the mass action of amino-acids acting as stimuli upon the cellular protoplasm.*







ANIMAL CALORIMETRY.

SIXTH PAPER.

THE INFLUENCE OF MIXTURES OF FOOD-STUFFS UPON METABOLISM.¹

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I. INTRODUCTION.

It was recognized by Rubner in his book *Die Gesetze des Energieverbrauchs* (p. 410) that, when mixtures of food-stuffs were given, the total heat production in the dog was not the sum of

¹ The urinary analyses of the fifth and sixth papers were accomplished by Miss Mary B. Wishart.

the increases which each food-stuff would have brought about had it been given alone, but was considerably less. Rubner explained this on the theory that the small amounts of protein ingested replaced that metabolized in fasting and, therefore, did not exert a specific dynamic action. He added that he did not deny the possibility of another explanation, it being conceivable that mixtures reacted specifically, or that one material, to a certain extent, took over the function of another.

II. EXPERIMENTAL PART.

The following experiments, for the most part originally planned to show the effect of amino-acids in metabolism, throw a definite light upon this subject. It was found that a mixed diet almost or quite masked the effects of ingesting amino-acids. Therefore, the method of giving these substances in Liebig's extract as described in the last paper was resorted to. The detailed results of all the experiments are shown in Table I.

A. The standard diet.

The standard diet was a mixture consisting of 100 grams of biscuit meal, 20 grams of lard and 100 grams of meat, to which were added 10 grams of bone ash. It was given daily (with a few exceptions) between October and May and sufficed to maintain Dog II at a nearly constant weight of 9.5 kgm. The composition of the lard was estimated but analyses were made of the biscuit meal and the meat of beef heart. The following table has been computed as a result of these analyses.

Composition of the standard diet.

DIET	GRAMS				CALORIES			
	N	Protein	Fat	Carbo- hydrate	Protein	Fat	Carbo- hydrate	Total
100 grams biscuit meal.	2.25	14.18	*4.79	73.0	62.4	44.5	299.5	400.4
20 grams lard.....			19.00			178.6		178.6
100 grams meat.....	3.00	18.25	*3.00		75.0	28.2		103.2
Total.....	5.25	32.43	26.79	73.0	137.4	251.3	299.5	688.2
Per cent.....					20	36	44	

* Ether extract.

This diet, which contained 688.2 calories or 1380 per square meter of surface per day or 28.7 calories per hour, was needed to maintain Dog II during six months in equilibrium of substance. The dog, for the most part, lived in a dog-room at a variable temperature and exercised freely; his energy production, asleep and eighteen hours after food ingestion, was but 16.2 calories an hour or 388.8 calories and 784 per square meter of surface per day at an environmental temperature of 26° to 27°. This shows that an increase of 70 per cent takes place in the metabolism of the dog, which is due to the effects of food ingestion, to mechanical work and to thermal influences encountered during twenty-four hours. During one period, on the 21st, 22nd and 23rd of November, the urine of the day was collected and found to contain 4.44, 4.27 and 4.44 gram of nitrogen or about 0.18 gram per hour.

The following paper concerns itself with the effects produced by this diet and by variations of it, during the first hours after its ingestion, the dog being quietly resting at an environmental temperature of 26° to 27°.

The results of the ingestion of the standard diet are here presented:

Experiments 5 and 10.

Standard diet at noon.

TIME	N IN URINE	NON-PROTEIN R.Q.	CALORIES		N IN URINE	NON-PROTEIN R.Q.	CALORIES	
			Cal- culated	Found			Cal- culated	Found
*10.00-11.00 a.m. Intermission					0.133	0.86	14.54	15.45
1.00-2.00 p.m.	0.194	0.90	23.37	23.41	0.182	0.93	22.82	21.13
2.00-2.00	0.230	0.96	22.78	22.85	0.215	0.89	24.87	22.77
3.00-4.00					0.208	0.98	23.01	22.05
Total			46.15	46.26			70.70	65.95
Per hour			23.08				23.56	

* Details in third paper.

Following the ingestion of the standard diet, the metabolism during the second to fourth hours shows an average heat production of 23.3 calories per hour. Since the basal metabolism has proved to average 16.2 calories per hour, the increase amounted

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to 7.1 calories or 44 per cent. From the respiratory quotient it is evident that the greater part of the oxidative processes was concerned with the metabolism of carbohydrate.

The urinary analyses. Following the ingestion of the standard diet at noon, the urine was analyzed in hourly periods with the following results:

Elimination of total N in grams after standard diet at noon.

TIME	NOV. 23	NOV. 27	NOV. 29	JAN. 8	DEC. 12 EXP. 5	DEC. 13	JAN. 9 EXP. 10
12.00-1.00	0.146	0.154	0.209	0.154		0.187	
1.00-2.00	0.142	0.174	0.202	0.182		0.194	
2.00-3.00	0.239	0.225	0.247	0.215		0.230	
3.00-4.00	0.229	0.241	0.255	0.208		0.240	
4.00-5.00	0.251	0.36†	0.282	0.236		0.250	
Total.....	1.007	1.155	1.195	0.995	1.081*	1.101	0.932†
* 12 to 5.15 p. m. † 12 to 5.09 p. m.							

It is evident; from this, that the hourly nitrogen output shows a constantly rising tendency. The values assumed for the calorimeter periods are approximate only, but it has already been set forth that a difference of 0.042 gram of nitrogen per hour makes almost no difference in the calculation of the heat production, and this latter is the essential point on the days when the calorimeter was used.

When 100 grams of meat were given alone instead of with 100 grams of biscuit meal and 20 grams of fat, the excretion of nitrogen in the urine was much more rapid as appears below (see also fifth paper):

April 9, 1912.	Total N.
12.00-1.00.....	0.208
1.00-2.00.....	0.343
2.00-3.00.....	0.397
3.00-4.00.....	0.334
4.00-5.00.....	0.407
Total.....	1.689

This can be due (1) to a more rapid absorption of protein or (2) to a more rapid destruction of amino-acids within the organism in the absence of carbohydrates.

B. The standard diet minus 67 grams of meat.

Beginning December 2, only 33 grams instead of 100 grams of meat were for a time added to the standard diet. On December 6 the metabolism was determined with the following results:

*Experiment 4.**Standard diet minus 67 grams of meat at noon.*

TIME	N IN URINE	NON-PROTEIN R.Q.	CALORIES	
			Calculated	Found
1.00-2.00	0.121	0.90	22.61	20.47
2.00-3.00	0.121	0.89	22.12	21.81
3.00-4.00	0.185	0.91	23.69	22.99
Total			68.42	65.27
Per hour.....			22.80	.

Again, the greater part of the non-protein calories is obtained from carbohydrate. The metabolism is increased from the basal value of 16.2 calories to 22.8, a rise of 6.6 calories or 41 per cent.

The metabolism is only 2 per cent less than the average metabolism after giving the full standard diet. The withdrawal of 67 grams of meat is, therefore, almost without influence on the metabolism, although it has been shown in paper five that 100 grams of meat ingested alone caused the hourly metabolism to rise from 16.2 to 20.2 calories, an increase of 4 calories or 25 per cent.

The urinary analyses. See next section.

C. The standard diet minus 67 grams of meat plus 20 grams of glutamic acid.

In this experiment, 20 grams of glutamic acid were added to the diet last employed so that the 1.9 grams of N contained in the glutamic acid might replace the 2 grams of N contained in the 67 grams of meat withdrawn from the standard diet. The results of the calorimeter experiments were as follows:

Experiment 6.

Standard diet minus 67 grams of meat plus 20 grams of glutamic acid at noon.

TIME	N IN URINE	NON-PROTEIN R.Q.	CALORIES	
			Calculated	Found
1.00-2.00	0.135	0.95	22.32	22.42
2.00-3.00	0.184	0.95	22.30	22.37
3.00-4.00	0.205	0.95	23.26	23.08
4.00-5.00	0.239	0.96	22.92	21.62
Total.....			90.80	89.49
Average.....			22.70	

The heat production is mostly at the expense of carbohydrate. The addition of glutamic acid was without influence upon the metabolism, which was measured as 22.7 calories per hour in contrast with 22.8 calories obtained before. This accords with the results shown in paper five, which demonstrated that glutamic acid exerted no influence upon heat production.

The urinary analyses. The analyses of the urines eliminated after giving the standard diet minus 67 grams of meat with and without 20 grams of glutamic acid are shown below.

Total N in urine after standard diet minus 67 grams of meat.

TIME	DEC. 4	DEC. 5 EXP. 4	DEC. 6	PLUS 20 GRAMS GLU- TAMIC ACID	
				DEC. 14	DEC. 15 EXP. 6
12.00-1.00	0.150		0.101	0.107	
1.00-2.00	0.135		0.122	0.185	
2.00-3.00	0.184		0.122	0.197	
3.00-4.00	0.205		0.185	0.250	
4.00-5.00	0.239		0.157	0.275	
Totals.....	0.913	0.642*	0.687	1.014	0.870†

* 12 to 5.06.

† 12 to 5.10.

The value obtained on December 15 was not sufficient to indicate with certainty that there was any extra excretion of nitrogen during five hours after the ingestion of the diet containing 20

grams of glutamic acid. The urine of the period contained 0.807 gram, of which 93 per cent was urea plus ammonia nitrogen.

From January 2 to 4, the standard diet was given at six p.m.; at noon on January 3, 50 grams of dextrose were ingested and two days later 50 grams of dextrose plus 20 grams of glutamic acid were administered. The following table shows the quantity of total nitrogen in the urine.

TIME	JANUARY 3	JANUARY 5
	50 GRAMS DEXTROSE	50 GRAMS DEXTROSE + 20 GRAMS GLUTAMIC ACID
12.00-1.00		0.067
1.00-2.00		0.111
2.00-3.00		0.198
3.00-4.00		0.188
4.00-5.00		0.149
Total	0.7273*	0.713

* 12 to 5.20.

Since the nitrogen of glutamic acid readily appears in the urine of a phlorhizinized dog or in the urine of a dog which has been given it with Liebig's extract, it seems that, when given with sugar, it must either (1) spare protein from destruction or (2) form a synthetic compound with carbohydrate either with glucose or with glycogen within the organism. This accords with the work of Lüthje, Murlin and others (see second paper).

In the calculation of those experiments in which glutamic acid was given with a mixed diet, the N elimination was attributed to protein metabolism.

Summarizing the total nitrogen excretion for five hours thus far discovered, the following results are found:

DATE	TIME	DIET	TOTAL N
Jan. 30, 1912	12.00-5.00	No food	0.650
Apr. 9, 1912	12.00-5.00	No food	0.660
Nov. 23, 1911	12.00-5.00	Standard diet	1.007
Nov. 27, 1911	12.00-5.00	Standard diet	1.155
Nov. 29, 1911	12.00-5.00	Standard diet	1.195
Nov. 12, 1911	12.00-5.15	Standard diet	1.081
Nov. 13, 1911	12.00-5.00	Standard diet	1.101
Jan. 8, 1912	12.00-5.00	Standard diet	0.995
Jan. 9, 1912	12.00-5.09	Standard diet	0.932
Dec. 4, 1911	12.00-5.00	Standard diet—67 grams meat	0.913
Dec. 5, 1911	12.00-5.06	Standard diet—67 grams meat	0.642
Dec. 6, 1911	12.00-5.00	Standard diet—67 grams meat	0.687
Dec. 14, 1911	12.00-5.00	Standard diet — 67 grams meat + 20 grams glutamic acid	1.014
Dec. 15, 1911	12.00-5.10	Standard diet — 67 grams meat + 20 grams glutamic acid	0.870

These experiments, by themselves, would confirm Rubner's view that the specific dynamic action of protein does not appear when protein is added to a mixed diet, because the added protein merely tends to replace the ordinary wear and tear quota of protein metabolism and is not quickly destroyed as happens when it is ingested alone. Hence, small variations in the amount of protein ingested would cause no change in the metabolism, as indeed these experiments tend to indicate.

The following work, however, establishes another view-point.

D. One-half portion of the standard diet.

It has been shown that the ingestion of the standard diet with its 100 grams of meat or the same with only 33 grams, or, 33 grams of meat plus 20 grams of glutamic acid, resulted in the same metabolism. It was decided to reduce the quantity of food that the action of the amino-acid in metabolism might be more strongly revealed. To this end, the standard diet was divided into two portions, Portion I and Portion II, each containing 50 grams of biscuit meal, 10 grams of lard and 5 grams of bone ash; in addition, Portion I contained 33 grams of meat while Portion II contained 67 grams of meat. Portion I was used exclusively as the diet for the experimental period and was given at noon daily, while Portion II was given at 6 p.m.

E. Portion I.

The influence of a half-portion of the standard diet known as Portion I, is shown in the following experiments:

*Experiments 25 and 27.**Portion I at noon.*

TIME	N IN URINE	NON-PROTEIN R.Q.	CALORIES		N IN URINE	NON-PROTEIN R.Q.	CALORIES	
			Cal- culated	Found			Cal- culated	Found
1.00-2.00	0.202	0.90	22.59	22.75	0.136	0.91	20.71	21.13
2.00-3.00	0.260	0.84	24.39	23.46	0.161	0.92	20.77	21.44
3.00-4.00	0.278	0.80	20.54	20.20	0.213	0.80	21.95	19.69
4.00-5.00	0.248	0.91	18.24	18.70	0.188	0.96	17.86	20.04
5.00-6.00					0.188	0.86	21.88	20.25
Total.....			85.76	85.11			103.17	102.55
Per hour.....			21.44				20.63	

Since the dog moved during a period of eight minutes, between two and three o'clock in Experiment 25, thereby causing the metabolism to increase 2 calories during this hour, the value 20.6 calories per hour, as found in Experiment 27, may be accepted as the true level of metabolism during the second to sixth hour after the ingestion of Portion I. The increase above the basal value of 16.2 calories was, therefore, 4.4 calories or 27 per cent per hour. For the whole period, the increase amounted to 22.7 calories. It may here be recalled that 50 grams of dextrose given to this same dog, increased the metabolism 20 per cent in the second hour after its ingestion, but it caused a total increase of only 8.8 calories. Portion I, consisting of 50 grams of biscuit meal, 33 grams of meat and 10 grams of fat, has a more powerful and more prolonged influence over the production of heat. To what can this be attributed? In part perhaps to the more prolonged period of absorption due to the time required for the digestion of starch. This is illustrated by the fact that the respiratory quotient indicates that carbohydrate is not so much a dominating factor in the metabolism as when 50 grams of dextrose are

ingested. The influence of other factors will be made clear in later experiments.

The urinary analyses. The following analyses were made of the urine after giving Portion I to the dog:

Urinary Analyses after Portion I at noon.

TIME	FEBRUARY 12		FEBRUARY 13		MARCH 5		MARCH 19	
	TOTAL N	UREA + NH ₄ -N	TOTAL N	UREA + NH ₄ -N	TOTAL N	UREA + NH ₄ -N	TOTAL N	UREA + NH ₄ -N
12.00-1.00	0.177	0.168	0.129	0.113	0.168	0.167	0.132	0.107
1.00-2.00	0.202	0.187	0.136	0.126	0.208	0.192	0.199	0.177
2.00-3.00	0.260	0.251	0.161	0.148	0.324	0.317	0.225	0.201
3.00-4.00	0.278	0.255	0.213	0.201	0.274	0.250	0.209	0.182
4.00-5.00	0.248	0.225	0.188	0.179	0.244	0.223	0.197	0.177
5.00-6.00					0.218	0.195	0.187	0.177
Total.....	1.165	1.086	0.827	0.767	1.436	1.344	1.149	1.021
Per cent.....		93		93		93		88

On February 14, in Experiment 25, the urinary nitrogen between 12.00 and 5.06 p.m. amounted to 1.07 grams, as against 1.19 grams, based on the figures obtained on February 12. On February 27, in Experiment 27, the urinary nitrogen was 1.039 grams between 12 and 6.06 p.m. as against 1.035 grams of nitrogen calculated for the same period of time on the basis of the figures obtained on February 13. These figures were adopted on the respective calorimeter days.

F. 50 grams of biscuit meal.

It was deemed of interest to note the effect of a part of Portion I. Whenever a part of the standard diet was given at noon, the remainder was always ingested at 6.00 p.m. the same day. The influence of the ingestion of 50 grams of biscuit meal given alone is shown in the following:

*Experiment 30.**50 grams of biscuit meal at noon.*

TIME	N IN URINE	NON-PROTEIN R.Q.	CALORIES	
			Calculated	Found
1.00-2.00	0.182	1.00	18.75	18.54
2.00-3.00	0.182	0.96	18.58	18.15
3.00-4.00	0.182	0.98	19.31	18.69
4.00-5.00	0.182	0.88	19.95	18.35
Total			76.59	73.73
Per hour.....			19.15	

It is evident from the above, that the 50 grams of biscuit meal induces a metabolism of about 19.15 calories per hour, in contrast with 16.2 calories without food and 20.6 calories with Portion I.

The urinary analyses. On February 23, 50 grams of biscuit meal were given at noon and the urine was collected in hourly periods and was analyzed with the following results:

Time.	Total N.
12.00-1.00	0.104
1.00-2.00	0.149
2.00-3.00	0.160
3.00-4.00	0.146
4.00-5.00	0.154
Total	0.713

On the day of the experiment, however, the urine between noon and 5.05 p.m. contained 0.930 gram N or 0.182 per hour. The comparative constancy of the results in hourly periods between one and five o'clock in the first experiment, led to the apportionment of the nitrogen of the calorimeter period equally among the several hours of the calorimeter experiment.

G. 50 grams of biscuit meal plus 10 grams of lard.

The results obtained after giving 50 grams of biscuit meal plus 10 grams of lard are given below:

*Experiment 31.**50 grams of biscuit meal plus 10 grams of lard.*

TIME	N IN URINE	NON-PROTEIN R.Q.	CALORIES	
			Calculated	Found
1.00-2.00	0.140	0.90	17.37	17.99
2.00-3.00	0.166	0.93	19.79	18.62
3.00-4.00	0.166	0.90	20.09	19.47
Total			57.25	56.08
Per hour			19.08	

The result of this experiment shows that *the heat production is uninfluenced by the addition of 10 grams of lard to 50 grams of biscuit meal*. The respiratory quotient is lowered by the ingestion of fat but Rubner's isodynamic law holds true, and the total heat production is not increased.

The urinary analyses. On February 28, the urine showed the following analytical results after giving 50 grams of biscuit meal plus 10 grams of lard:

Time.	Total N.
12.00-1.00	0.1306
1.00-2.00	0.2078
2.00-3.00	0.2585
3.00-4.00	0.2503
Total	0.8470

Since the urine of February 29 during the period from noon till 4.07 p.m. contained only 0.677 gram of nitrogen, nitrogen values obtained after giving 50 grams of biscuit meal on February 27 were employed.

H. Analysis of the effect of Portion I.

The following table summarized the results obtained after giving Portion I and fractions thereof:

DIET	N IN URINE PER HOUR	CALCULATED PER HOUR
No food	0.132	16.2
50 grams biscuit meal	0.182	19.15
50 grams biscuit meal + 10 grams lard	0.164	19.08
50 grams biscuit meal + 10 grams lard + 33 grams meat	0.170	20.63

It is evident that the addition of a small quantity of fat has no influence on the heat production, whereas as small an amount of meat as 33 grams very considerably increases the metabolism. The increase in metabolism caused by meat ingestion is, however, in no way accompanied by an increased protein metabolism as indicated by comparison of the average hourly output of urinary nitrogen during the calorimeter periods. It has already been shown in the last paper, that 100 grams of meat (3 grams N) caused the production of 20.2 calories per hour in this dog, so that it may be that the amino-acids from 33 grams (1 gram N) of meat, together with those contained in 50 grams of biscuit meal (1.1 grams N), may be largely determinative of the height of metabolism (20.6 calories) when Portion I is given. This would be true if the cells were stimulated to increased oxidation by amino-acids. In presenting this idea, it is not forgotten that Rubner's interpretation, as regards the cause of the rise in heat production, also explains the phenomenon.

The truth of the matter, however, was to be laid bare after the completion of experiments involving the ingestion of amino-acids with Portion I.

I. 20 grams of glutamic acid plus Portion I.

As was to be expected from the former work, glutamic acid showed no power to increase metabolism when it was given with Portion I. The results of an experiment illustrating this is given here:

Experiment 26.

20 grams of glutamic acid plus Portion I at noon.

TIME	N IN URINE	NON-PROTEIN R.Q.	CALORIES	
			Calculated	Found
1.00-2.00	0.202	1.03	20.80	21.66
2.00-3.00	0.260	0.96	19.08	19.76
3.00-4.00	0.278	0.72	18.69	18.11
4.00-5.00	0.248	0.90	21.96	19.76
Total			80.53	79.29
Per hour.....			20.13	

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The value 20.13 calories per hour agrees well with 20.6 calories found with Portion I alone on February 19.

The urinary analyses. The same condition of affairs as was found to exist after giving glutamic acid with the standard diet, was revealed in the analysis of the urine after giving Portion I with glutamic acid, that is to say, there was no certain increase in the amount of nitrogen in the urine. This is shown in the following:

Urinary analyses after giving Portion I plus 20 grams of glutamic acid at noon.

TIME	FEBRUARY 15		FEBRUARY 16	
	TOTAL N	UREA + NH_3 N	TOTAL N	UREA + NH_3 N
10.00-11.00	0.103		0.139	
Intermission				
12.00-1.00	0.123	0.107		
1.00-2.00	0.157	0.143		
2.00-3.00	0.208	0.184		
3.00-4.00	0.180	0.156		
4.00-5.00	0.282	0.262		
Total	0.950	0.852	1.234*	1.130
Per cent.		89		91

* Urine from noon to 5.08 p. m.

Although the urine obtained on the calorimeter day, contained more nitrogen than on the day previous, it did not contain more than on February 12, when Portion I alone was given. The hourly figures of February 12 were, therefore, adopted as representing the protein metabolism of February 16. On the latter date 7.15 grams of N were given including that contained in Portions I and II and 20 grams of glutamic acid, while during the twenty-four hours from noon till noon of the next day only 3.79 grams were eliminated in the urine. It does not seem possible that the explanation of this is due to non-absorption of glutamic acid, for given with Liebig's extract it is freely absorbed; nor is it probable that it can be stored without change, for this result was obtained on the second consecutive day of its administration.²

² The matter has not been followed to a conclusion, but it may be possible that glutamic acid favors synthetic construction of new protein.

J. 20 grams of alanine plus Portion I.

In paper five it was shown that the ingestion of 20 grams of alanine with Liebig's extract of meat caused a rise of heat production from 16.2 calories to 19.2 calories per hour extending through the fifth hour. To the same dog, Portion I with 20 grams of alanine was administered with the following results:

*Experiments 34 and 32.**20 grams of alanine + Portion I at noon.*

TIME	PROTEIN N	AMINO-ACID N	NON-PROTEIN R.Q.	CALORIES				
				Protein	Amino-acid	Non-protein	Total	
							Calculated	Found
1.00-2.00	0.212	0.047	0.96	5.63	0.91	12.03	18.57	21.19
2.00-3.00	0.225	0.169	0.86	5.98	3.27	11.29	20.54	20.84
3.00-4.00	0.209	0.242	1.05	5.55	4.67	9.12	19.34	21.68
4.00-5.00	0.197	0.220	0.83	5.21	4.25	11.07	20.53	21.08
5.00-6.00	0.187	0.136	0.92	4.95	2.62	13.92	21.49	22.76
Total				27.32	15.72	57.43	100.47	107.55
Per hour							20.09	

The experiments presented here should be compared with Experiments 25 and 27 when Portion I was given without the addition of alanine and in which the metabolism was 21.4 and 20.6 calories per hour. *There is no increase in metabolism due to the addition of 20 grams of alanine to Portion I.* In paper five, it was estimated that 15.46 calories were liberated from alanine metabolized in four hours after the administration of 20 grams of the substance in Liebig's broth. In the present experiment, the estimated increase is 15.7 calories for five hours. *Although alanine was certainly metabolized, it exerted no "specific dynamic" influence when given with the mixed diet which produced a total metabolism of 20.6 calories per hour, although administered alone it raised the metabolism from the basal value of 16.2 to 19.2 calories per hour.*

The urinary analyses. After adding 20 grams of alanine to Portion I, the urinary nitrogen nearly doubled in quantity although no such effect was obtained with glutamic acid. The results are given below:

200 Metabolism of Mixtures of Food-Stuffs

Urinary analyses after giving Portion I plus 20 grams of alanine at noon.

TIME	MARCH 1*		MARCH 4	
	TOTAL N	UREA + NH ₃ N	TOTAL N	UREA + NH ₃ N
12.00-1.00	0.234	0.181	0.260	0.218
1.00-2.00	0.234	0.181	0.260	0.174
2.00-3.00	0.396	0.289	0.466	0.369
3.00-4.00	0.396	0.289	0.519	0.409
4.00-5.00	0.460	0.369	0.484	0.379
5.00-6.00	0.460	0.369	0.338	0.295
Total	2.180	1.678	2.327	1.844
Per cent.....		77		79

* The urine of this day was obtained in periods of two hours each.

On March 7, Experiment 32, and on March 9, Experiment 34, when the dog was in the calorimeter, the following urinary analyses were made and are compared with the figures obtained on March 4, as given above:

DATE	TIME	TOTAL N	UREA + NH ₃ N	PER CENT
March 7	12.00-6.00	2.564	1.995	78
March 4	12.00-6.00	2.327	1.844	79
March 9	12.00-4.00	1.397	0.971	70
March 4	12.00-4.00	1.505	1.170	79

The nitrogen figures found on March 4 appear, therefore, to be a trustworthy indication of the results on the days when the dog was in the calorimeter. To determine the hourly protein metabolism, the nitrogen figures obtained when Portion I was given on March 19 were adopted. To determine the metabolism of alanine, the difference between the urea plus ammonia nitrogen figures obtained on March 19 after giving Portion I and the urea plus ammonia nitrogen after giving Portion I plus alanine on March 4, was calculated, and with the following results:

Time.	N of alanine metabolized.
12.00-1.00.....	0.122
1.00-2.00.....	0.047
2.00-3.00.....	0.169
3.00-4.00.....	0.242
4.00-5.00.....	0.220
5.00-6.00.....	0.136
Total.....	0.936

The figures were adopted in the hourly computations in Experiments 32 and 34. The objections to the method were discussed in paper five.

K. 20 grams of glycocoll plus Portion I.

The results obtained after giving 25 grams of glycocoll were shown in paper five. The metabolism rose from 16.2 calories to 21.8 and 22.8 calories per hour during the second to fifth hours after its ingestion. In the following experiments, 20 grams of glycocoll were given with Portion I with the following results:

Experiments 35 and 37.

20 grams of glycocoll plus Portion I at noon.

TIME	URINARY PROTEIN N	URINARY AMINO-ACID N	NON-PROTEIN R.Q.	CALORIES				
				Protein	Amino- acid	Non- protein	Total	
							Cal- culated	Found
1.00-2.00	0.199	0.091	0.95	5.41	0.90	15.67	21.98	22.52
2.00-3.00	0.225	0.090	0.89	5.98	0.89	16.44	23.31	21.80
3.00-4.00	0.209	0.130	0.86	5.49	1.28	18.15	24.92	22.13
4.00-5.00	0.197	0.222	0.93	5.21	2.20	13.75	21.16	20.44
Total				22.09	5.27	64.01	91.37	86.89
Per hour							22.85	21.72
1.00-2.00	0.199	0.091	0.91	5.41	0.90	16.86	23.17	22.05
2.00-3.00	0.225	0.090	0.89	5.98	0.89	17.23	24.10	22.25
3.00-4.00	0.209	0.130	1.06	5.49	1.28	14.26	21.03	22.59
4.00-5.00	0.197	0.222	0.93	5.21	2.20	15.28	22.69	21.99
Total				22.09	5.27	63.63	90.99	88.88
Per hour							22.75	22.22

In order to interpret these experiments, comparisons are made below between the results obtained with (1) Portion I alone, (2) Portion I plus 20 grams of glycocoll and (3) 25 grams of glycocoll alone (see paper five).

The influence of adding glycerol to a mixed diet.

EXP. NO.	DATE	TIME	FOOD	CALORIES	
				Calculated	Found
25	Feb. 14	1 00-5.00	Portion I	85.72	85.11
27	Feb. 19	1.00-5.00	Portion I	81.29	82.30
35	Mar. 13	1.00-5.00	Portion I + 20 grams glycerol	91.37	86.89
37	Mar. 19	1.00-5.00	Portion I + 20 grams glycerol	90.99	88.88
41	Mar. 29	1.00-5.00	25 grams glycerol	87.33	82.06
44	Apr. 4	1.00-5.00	25 grams glycerol	91.21	85.66

The phenomenon after glycerol ingestion of a constantly higher calculated metabolism than that observed calorimetrically is apparent from these figures, but the cause is not easily explained. It becomes evident, from consideration of this table, that *the addition of glycerol to a mixed diet may slightly raise the heat production but it does not raise it higher than if glycerol were alone ingested. This affords the proof that the amino-acid, glycerol, acts in an entirely different manner in raising the heat production than do carbohydrate and fat.* Glycerol was almost as completely oxidized when given with Portion I as when given alone, the respective calories from glycerol being at least 5.27 in the first instance (see urinary analyses) and 6.08 in the last. From this it may be concluded that the carbohydrate ingested had no influence upon the metabolism of glycerol in the organism. Neither alanine nor glycerol have shown any appreciable "specific dynamic" influence when added to a mixed diet.

The explanation is the following. The amino-acids act as stimuli upon the cells, raising their power to metabolize (papers two and five). They may act instead of nerve stimuli when increased heat production is demanded in the presence of external cold, "the chemical regulation of temperature" of Rubner. The energy liberated in response to these stimuli may be supplied by carbohydrate or by fat.

When fat and carbohydrate are given separately or together, the heat production may also be increased on account of the increase in the quantity of materials available for the nutrition of

the cells in the sense of Voit. With the cessation of absorption and the return of the blood to its composition before food was given, the metabolism falls to the basal value as shown in paper three.

The increased heat production after giving carbohydrate, must be accompanied by increased movement of the cell particles and cannot be due to extra-cellular oxidations, for amino-acids which act as stimuli when ingested, do not increase the metabolism under the conditions attendant upon the absorption of a mixed diet. The absorbed amino-acids, under these circumstances, merely encounter a level of cellular oxidation, which they, themselves, alone would induce, but to which they cannot further contribute.

The urinary analyses. These were made exactly as in the experiments with glyocoll, and the calculations were based on exactly the same principles. The results were as follows:

Urinary analyses after giving Portion I plus 20 grams of glyocoll.

TIME	MARCH 11	
	TOTAL N	UREA + NH ₃ N
12.00-1.00	0.233	0.194
1.00-2.00	0.340	0.268
2.00-3.00	0.324	0.291
3.00-4.00	0.337	0.312
4.00-5.00	0.434	0.399
5.00-6.00	0.441	0.418
Total.....	2.109	1.882
Per cent		89

On March 13, Experiment 35, and on March 16, Experiment 37, when the dog was in the calorimeter, the urine was analyzed and the results compared with those obtained on March 11 are given below.

DATE	TIME	TOTAL N	UREA + NH ₃ N	PER CENT
March 13	12.00-5.09	1.895	1.860	98
March 11	12.00-5.09	1.734	1.527	88
March 16	12.00-5.08	1.859	1.727	93
March 11	12.00-5.08	1.728	1.521	88

If one employs the urea plus ammonia N found on March 19 as the basis of normal value when Portion I alone was given, then the increase in the urea plus ammonia nitrogen on March 11, due to glycocoll metabolized, would be:

Time.	N of glycocoll metabolized.
12.00-1.00.....	0.087
1.00-2.00.....	0.091
2.00-3.00.....	0.090
3.00-4.00.....	0.130
4.00-5.00.....	0.222
Total.....	0.620

These figures, which were adopted, must be regarded as minimal values on account of the very low value of urea plus ammonia nitrogen found on March 11.

On March 19, when Portion I alone was given, the urea plus ammonia nitrogen amounted to 0.871 gram calculated between noon and 5.09 p.m. Deducting this value from the urea plus ammonia nitrogen found on the calorimeter days, March 13 and 16, one obtains 0.989 and 0.859 gram of glycocoll N metabolized instead of 0.620 adopted for five hours. Recalculation of the metabolism on the basis of the higher glycocoll figures, would have tended to reduce the "calories calculated" by an amount not exceeding 1 calorie during the four hours of the experiment.

III. GENERAL STATISTICS.

The following is a statistical summary of the results obtained during the first hours after the ingestion of the following diets:

EXP. NO.	DIET	CALORIES CALCULATED PER HOUR
	No food	16.2
5	100 g. biscuit meal, 100 g. meat, 20 g. lard	23.1
10	100 g. biscuit meal, 100 g. meat, 20 g. lard	23.6
4	100 g. biscuit meal, 33 g. meat, 20 g. lard	22.8
6	100 g. biscuit meal, 33 g. meat, 20 g. lard, 20 g. glutamic acid	22.7
25	50 g. biscuit meal, 33 g. meat, 10 g. lard	21.4
27	50 g. biscuit meal, 33 g. meat, 10 g. lard	20.6
30	50 g. biscuit meal	19.1
31	50 g. biscuit meal, 10 g. lard	19.1
26	50 g. biscuit meal, 33 g. meat, 10 g. lard, 20 g. glutamic acid	20.1
32 and 33	50 g. biscuit meal, 33 g. meat, 10 g. lard, 20 g. alanine	20.1
35	50 g. biscuit meal, 33 g. meat, 10 g. lard, 20 g. glycocoll	22.8
37	50 g. biscuit meal, 33 g. meat, 10 g. lard, 20 g. glycocoll	22.7

IV. SUMMARY.

1. A dog was given daily a maintenance or standard diet during a period of about six months. When the diet was given at six p.m., the basal metabolism on the following day, from noon till four o'clock, was found to be about 16.2 calories per hour. The ingestion of the standard diet, consisting of 100 grams of biscuit meal, 100 grams of meat and 20 grams of lard, brought about, during the first four hours, an average hourly heat production of 23.3 calories, an increase of 44 per cent above the basal value. Reduction in the quantity of meat ingested in the diet from 100 to 33 grams, and also addition of 20 grams of glutamic acid to this modification of the standard diet, had no pronounced influence upon the heat production.

2. Certain portions of the standard diet were given at noon and the rest at six in the afternoon. When this was done, it was found that 50 grams of biscuit meal induced a metabolism of 19.1 calories per hour, which was unchanged by the addition of 10 grams of fat to the diet. However, the further addition of 33 grams of meat to the mixture of biscuit meal and lard caused

the metabolism to rise to 20.6 calories per hour. Since it has been found in other experiments that 100 grams of meat alone increased the metabolism from 16.2 to 20.2 calories per hour, it appeared possible that the amino-acids of the meat and of the biscuit meal might be the determining factor as to the height of the metabolism, when Portion I (50 grams biscuit meal, 33 grams meat, 10 grams lard) was given.

3. Portion I given with 20 grams of glutamic acid, showed the latter to be without influence, 20.1 calories being produced per hour. Given with 20 grams of alanine, the metabolism was also uninfluenced, remaining at 20.1 calories per hour, although 20 grams of alanine given alone raises the metabolism from 16.2 to 19.2 calories per hour. When, however, Portion I was given with 20 grams of glycocoll, the metabolism rose to 22.7 and 22.8 or to the same level found in experiments in which 25 grams of glycocoll were given alone.

4. It seems that 20 grams of alanine given alone induces a metabolism of 19.2 calories, but given with a mixed diet which, of itself, establishes a metabolism of 20.6 calories, it is without effect. When 25 grams of glycocoll are given alone the heat production reaches 22.8 calories per hour, and when 20 grams were given with Portion I the heat production rose to a calculated value of 22.7 and 22.8 calories. It appears, therefore, that, in this case, the metabolism rose to the height which glycocoll alone would have induced.

5. These results taken in connection with those of the foregoing papers, establish a more exact conception of metabolism. One may believe that a larger oxidation of fat and carbohydrate takes place when these substances are furnished to the cells in increased quantity during the absorption of food, as has been set forth in papers three and four. Addition of fat in moderate quantity, however, may not increase a metabolism already raised to a higher level by the ingestion of carbohydrate, Rubner's isodynamic law controlling. Addition of meat or of amino-acids to a mixed diet does not increase the metabolism of fat and carbohydrate unless the stimulus of the amino-acids would alone effect this result.

One may conclude that there are the following forms of metabolism in the quiet or sleeping dog excluded from thermal influences. (1) *A basal metabolism*, when the cells are nourished by a

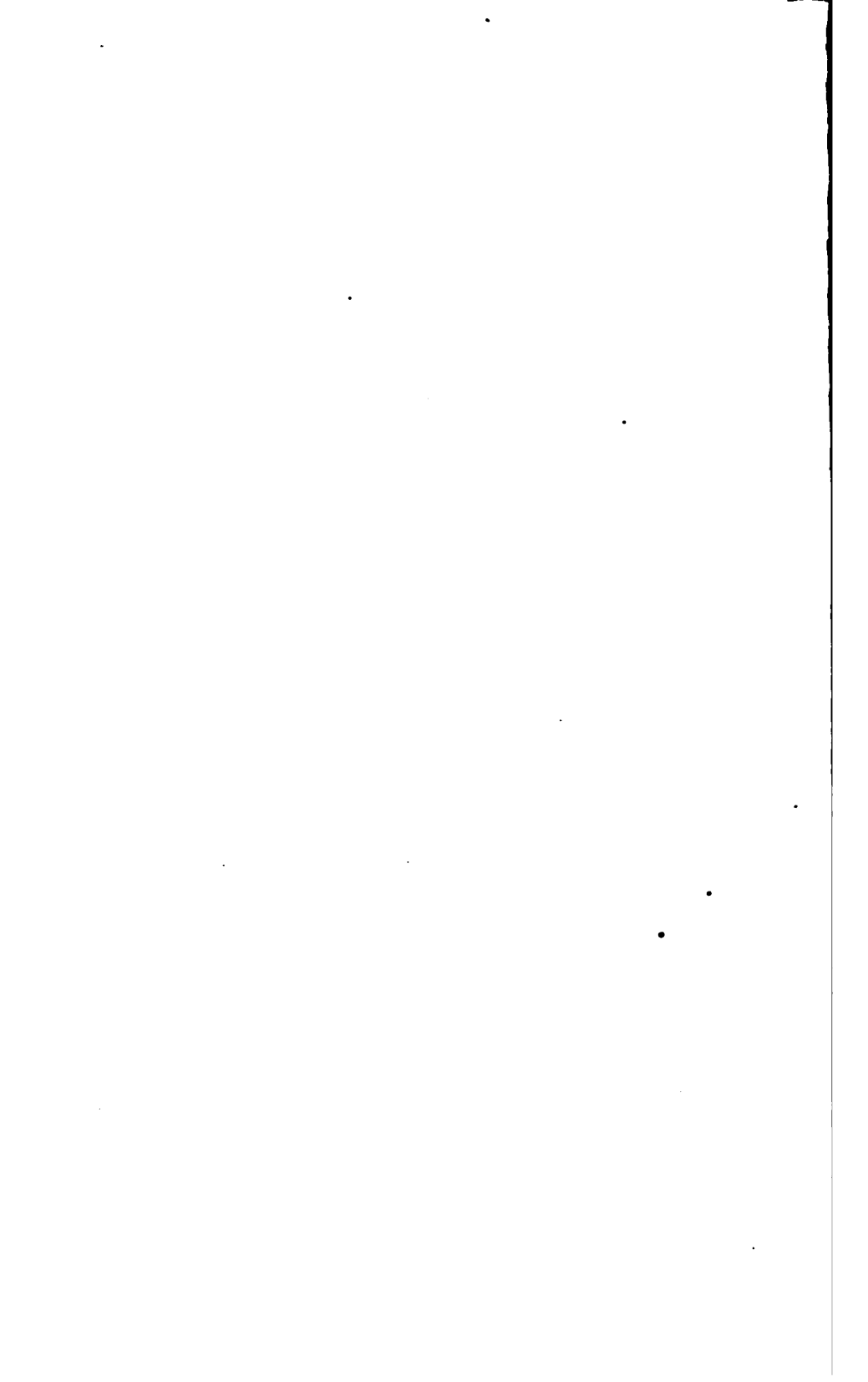
blood stream which does not receive food from the intestinal tract but the composition of which is regulated by the organs of the body; (2) *a metabolism due to plethora*, induced by an increased quantity of carbohydrate or fat metabolites in the blood on account of absorption from the intestine, and (3) *a metabolism due to the stimulus of amino-acids*. The metabolism of plethora and the metabolism of amino-acid stimulation cannot be added to each other; there is no summation of effect when both influences are brought into action simultaneously. In other words, the level of cellular oxidation induced by plethora is not further heightened by the stimulus of amino-acids, unless the latter alone would accomplish such increase in activity.

It is realized by the writer that the evidence here presented in favor of this conception of the manner of metabolism may be largely increased by further investigation, and he hopes that the future may hold opportunity for such work.









ON THE CREATINE METABOLISM OF THE GROWING PIG.¹

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In a series of recent papers Mendel and Rose² have shown that in fasting rabbits creatine appears in the urine in large amounts but disappears when the animal is fed a liberal supply of carbohydrate. They have also shown that, in dogs, after the store of glycogen is depleted by phlorhizin administration, creatine appears in the urine in significant amounts and when the glycogenic function is disturbed by phosphorus poisoning creatine appears in large amounts. Feeding protein alone, fat alone or protein and fat did not prevent the excretion of creatine in these animals. They conclude that there exists an intimate relation between the creatine metabolism and the carbohydrate metabolism and that protein metabolism of the exogenous type does not lead to the formation of creatine.

Howe, Mattill and Hawk³ state that in a dog no appreciable increase in the creatine content of the urine was observed during the first sixty days of fasting.

Rose⁴ has observed that creatine was present in the urine of children under fifteen years of age in twenty-five out of twenty-seven cases examined, but not in urines from older persons. Indeed it is well known that creatine is but a casual constituent of the urine of normal adult mammals. All investigators agree that creatine

¹ Published with the permission of the Director of the Wisconsin Experiment Station.

² Mendel and Rose: this *Journal*, x, pp. 213-264, 1911. A full discussion of the literature is given by them.

³ Howe, Mattill and Hawk: this *Journal*, xi, p. 97, 1912.

⁴ Rose: this *Journal*, x, p. 265, 1911.

appears in the urine of fasting men, rabbits and dogs but the amount present in the urine of the dog may be very little influenced by fasting.⁵

Folin and Denis⁶ have confirmed the observation of Rose respecting the presence of creatine in the urine of young children even when no creatine is taken in the food. They attribute the presence of creatine to the high protein content of the children's diet.

It is difficult to reconcile the fact that children excrete creatine with the theory of Mendel and Rose that creatine does not result from the metabolism of exogenous or of reserve protein, but only from endogenous metabolism. Well-fed children take so much carbohydrate as to make one doubt whether a sufficient carbohydrate hunger could ensue (during the night) to account for the relatively high values regularly found for creatine.

The present work was planned to throw light on whether creatine can originate from exogenous protein metabolism and, if so, what is the influence of the plane of protein intake on creatine

TABLE I.

Creatine and creatinine elimination during inanition in the pig.

Weight of pig, 85 pounds. Urine preserved with a solution of thymol in toluene. Starvation started January 19.

DATE	VOLUME OF URINE	TOTAL N	CREATININE	TOTAL CREATININE
<i>January</i>	<i>cc.</i>	<i>grams</i>	<i>gram</i>	<i>gram</i>
25	355	3.713	0.898	0.898
26	300	3.906	0.916	0.900
27	350	3.518	0.821	0.798
28	216	3.101	*	0.829
29	170	2.822	0.688	0.706
30	184	2.903	0.745	0.745
31	156	2.761	0.683	0.683
1	174	2.470	0.655	0.655
2	200	2.812	0.720	0.736

* Determination lost through the formation of difficultly soluble crystals on standing when preserved with picric acid in the volumetric flask.

⁵ Howe, Mattill and Hawk: *loc. cit.*; Underhill and Kleiner: this *Journal* iv, p. 165, 1908; Mendel and Rose: *ibid.*, x, p. 213, 1911, give a discussion of the literature of creatine and creatinine metabolism; Dorner: *Zeitschr. f. physiol. Chem.*, lii, pp. 239, 243, 1907.

⁶ Folin and Denis: this *Journal*, xi, p. 253, 1912.

excretion when the energy metabolized remains constant. Pigs were employed as experimental animals.

At the outset we were surprised to find that moderate fasting does not lead to creatine elimination in the pig. One of us has noted⁷ that after a few days on a diet of starch and salts the urine of growing pigs becomes creatine-free. We have conducted two experiments in which pigs were fasted for fourteen and sixteen days respectively without the appearance of appreciable amounts of creatine in the urine. Table I gives the results of one of these experiments. In the other case only the urines of the thirteenth to sixteenth days were examined and creatine was found to be absent.

Of the different species of animals observed the rabbit seems to be the most sensitive to fasting, a high nitrogen and creatine output appearing in two or three days. It is a significant fact that Mendel and Rose⁸ obtained the largest creatine elimination with the most poorly nourished rabbit during starvation. The dog is much less sensitive and from our observations it appears that the pig is still less so than the dog. The fact that a well-nourished pig can fast from fourteen to sixteen days without excreting appreciable amounts of creatine, sheds considerable light on the interpretation which should be put upon the observations on this point made upon other species.

The most plausible explanation of these results would seem to be the assumption of different degrees of ability to utilize fat as a source of energy. When the rabbit fasts, the total nitrogen rises rapidly indicating an increase in endogenous nitrogen catabolism.⁹ This does not happen in the dog except possibly in a slight degree and our own observations on fasting pigs have shown that the total nitrogen in the urine steadily falls to a very low level. On teleological grounds one might reason that, since the pig is one of the most efficient storers of fat, he might be expected to utilize it readily when necessary for energy production. This seems to be the case. The elimination of much creatine by the rabbit during fasting is unquestionably the result of increased tissue catabolism due to his inability to draw his supply of energy from other

⁷ McCollum: *Amer. Journ. of Physiol.*, xxix, p. 210, 1911.

⁸ This *Journal*, x, p. 222, 1911.

⁹ Cf. Mendel and Rose: *ibid.*

sources (fat). An endogenous origin of creatine seems therefore to be definitely established. In the dog, fat is oxidized or otherwise transformed for energy production with much greater facility than in the rabbit and the endogenous metabolism is therefore not greatly accelerated during starvation so long as fat is available. In the pig, since we find no creatine in the urine during the early stages of fasting (14-16 days), it would seem that fat is utilized so readily for energy production that no acceleration of tissue catabolism takes place. The small amount of creatine of endogenous origin, when the latter type of catabolism is at its minimum, is destroyed in the body. All observers agree that on a liberal carbohydrate diet no creatine is excreted by any of the species referred to above. Our experience, in accord with others, shows that there exists considerable irregularity in the excretion of creatine on a uniform diet. This has been attributed to a destruction of creatine by enzyme action. We have data ourselves tending to substantiate this idea. Urines preserved with both toluene and chloroform and incubated at 37° showed a regular and fairly rapid decrease in creatine and total creatinine content. These enzymes, on the basis of the work of Gottlieb and Stangassinger¹⁰ and of Rothman,¹¹ seem to be generally distributed through the animal tissues.

TABLE II.

Weight of pig, 66 pounds at beginning, 67 pounds at end of record. Diet, 825 grams corn meal; 13.85 grams N and 2902 calories. Calories per kilo, 97 (55 × creatinine N).

DATE	VOLUME OF URINE	N IN URINE	CREATININE	CREATINE	CREATINE AS PERCENTAGE OF TOTAL CREATININE
May	cc.	grams	gram	gram	
25	923	6.770	0.7672	0.0326	4.07
26	1170	7.500	0.9266	0.0	0.0
27	1230	7.380	0.7140	0.0	0.0
28	1230	7.257	0.7683	0.0	0.0
29	1210	8.107	0.8615	0.0	0.0
30	1570	9.263	0.5893	0.0448	7.11
31	1410	7.896	0.8184	0.0093	1.12

¹⁰ *Zeitschr. f. physiol. Chem.*, lii, p. 1, 1907.

¹¹ *Ibid.* lvii, p. 131, 1908.

One of the most interesting points brought out by the experiments of Mendel and Rose is that feeding protein (casein, egg albumen) to fasting rabbits does not decrease the excretion of creatine. Numerous isolated tests for creatine in the urines of pigs on diets supplying proteins from restricted sources indicate that in young growing pigs, of 40 to 65 pounds body-weight, on the same plane of protein and energy intake, creatine is sometimes present in large amounts and in other cases absent or nearly so. We were led by these observations to examine for suitable periods the urines of several pigs on a number of different diets and have observed that it is undoubtedly true that a high protein intake *from certain sources* leads to the elimination of creatine, but an equally high protein intake from another source may cause the excretion of but small amounts of creatine. Table II shows the record of a pig taking fifty-five times the nitrogen eliminated daily as creatinine (approximately ten times his endogenous requirement), the food nitrogen being derived wholly from corn meal (maize). The creatine output was very small, in fact only a casual constituent of the urine.

Table III shows the record of a pig taking nitrogen from corn meal, equivalent to twenty-seven and one-half times his creatinine nitrogen output. On ten out of sixteen days on this ration there was no creatine in the urine. When on July 27 the ration was changed to starch, linseed meal (nearly oil-free) and gluten meal,¹² although the nitrogen fed was somewhat less in amount, there was at once a marked rise in the creatine content of the urine. On August 8, the nitrogen intake was doubled, but during the following five days the creatine output was not in proportion to the increased protein intake. This may be due to the great retention of nitrogen for growth.

Table IV shows the record of another pig on the same diet, with an intake of fifty-five times his creatinine nitrogen elimination. The results are in accord with those in table III.

Table V shows the record of a pig on the same plane of protein intake as was the pig in table II (55 times the creatinine nitrogen), the ration being derived from equal parts of wheat, rolled oats

¹² Gluten meal is a by-product of starch manufacture and consists principally of the proteins of the corn endosperm.

Creatine Metabolism of the Pig

TABLE III.

Fig 26. Fed on corn since June 11. Weight, 46 pounds.

DATE	VOLUME OF URINE	TOTAL N IN URINE	CREATININE	TOTAL CREATININE	CREATINE AS PERCENTAGE OF TOTAL CREATININE	RATION FED
<i>July</i>	cc.	grams	grams	grams		
12	930	5.39	0.837	0.837	0.0	484 grams corn (7.93 grams N), 27.5 × creatinine N.
13	740	4.36	0.677	0.677	0.0	
14	690	4.41	0.703	0.703	0.0	
15	970	5.23	0.844	0.844	0.0	
16	1020	5.10	0.687	0.734	6.4	
17	925	5.18	0.702	0.767	8.4	
18	1080	4.86	0.613	0.738	16.9	
19	920	5.52	0.790	0.830	4.8	
20	830	5.23	0.699	0.710	1.5	
21	880	5.19	0.659	0.659	0.0	
22	910	4.46	0.608	0.608	0.0	
23	930	4.56	0.669	0.706	5.2	
24	930	4.83	0.687	0.687	0.0	
25	770	4.54	0.649	0.649	0.0	
26	900	5.67	0.771	0.771	0.0	Ration changed a.m. to 150 grams starch, 60 grams oil meal, 40 grams gluten meal (6.94 grams N).
27	1180	5.07	0.732	0.732	0.0	
28	1570	5.49	0.671	0.822	18.3	
29	1280	4.86	0.677	0.909	25.5	
30	1440	4.75	0.653	0.777	15.9	
31	1480	5.32	0.776	0.858	9.5	
<i>August</i>						
1	1210	4.72	0.621	0.750	17.1	
2	860	4.82	0.611	0.725	15.0	
3	1170	4.68	0.664	0.734	9.5	
4	1040	4.47	0.623	0.684	8.9	Ration changed a.m. to 50 grams starch, 120 grams oil meal, 80 grams gluten meal (13.88 grams N), 55 × creatinine N.
5	1140	5.36	0.654	0.779	16.0	
6	770	4.62	0.659	0.704	6.1	
7	1135	5.10				
8	1300	4.68	1.648	1.245	47.97	
9	740	6.58	0.636	0.710	10.42	
10	1210	8.11	0.709	0.850	16.58	
11	1055	8.23	0.708	0.841	16.81	
12	1180	7.91	0.674	0.770	12.47	

TABLE IV.

Fig 25. Fed on milk from June 11 to July 12. Weight, 75.5 pounds.

DATE	VOLUME OF URINE	TOTAL N IN URINE	CREATININE	TOTAL CREATININE	CREATINE AS PERCENTAGE OF TOTAL CREATININE	RATION FED
<i>July</i>	<i>cc.</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>		
14	730	6.86	0.939	1.066	11.9	200 grams starch, 49.2 grams gluten meal, 71.8 grams oil meal (8.5 grams N).
15	665	5.38	0.854	0.873	2.1	
16	1150	7.70	0.948	1.265	25.0	
17	920	6.25	0.919	1.127	18.4	
18	670	5.22	0.963	1.131	23.6	
19	1080	6.80	0.972	1.296	25.6	
20	770	5.39	0.876	1.093	19.8	
21	660	6.07	0.869	1.048	17.0	
22	810	5.75	0.840	0.972	13.5	
23	980	5.88	0.982	1.115	16.7	
24	800	5.84	0.863	1.166	25.9	
25	875	5.60	0.791	1.049	24.6	
26	820	6.06	0.983	0.983	0.0	
27	810	5.43	0.810	0.892	9.1	
28	1000	5.04	0.749	0.805	6.9	100 grams, additional starch.
29	1270	5.97	0.951	1.087	12.5	
30	790	4.66	0.591	0.676	12.6	
31	1140	6.95	1.061	1.282	17.2	
<i>August</i>						
1	1365	6.14	0.933	0.950	1.7	200 grams additional starch.
2	1360	5.71	0.928	0.940	1.2	
3	1350	5.80	0.922	0.922	0.0	
4	1040	5.51	0.876	1.002	12.5	
5	1060	5.93	0.922	1.003	8.0	
6	1085	5.56	0.848	1.125	24.6	
7	1050	5.67				
8	1150	5.63	0.923	1.124	17.88	Ration changed to 80 grams starch, 143 grams oil meal, 98 grams gluten meal (16.7 grams N).
9	1350	9.45	0.914	1.404	34.89	
10	1220	9.15	0.922	1.242	25.76	
11	1280	12.29	0.871	1.440	39.51	
12	1085	9.43	0.842	1.121	28.45	

and corn meal. There was regularly a high content of creatine in the urine.

With this relatively high nitrogen and very high energy intake creatine was constantly present in the urine on a creatine-free diet.

TABLE V.

Pig 15. Weight of pig 60 pounds at beginning, 67 pounds at end of record. Diet, 280 grams rolled oats, 280 grams wheat, 280 grams corn (N intake 16.48 grams, $55 \times$ creatinine N). Calories per kgm. (mean weight, 29 kgm.), about 106. Record for creatine excretion begins with the twenty-fifth day of feeding.

DATE	VOLUME OF URINE	N IN URINE	CREATININE	CREATINE	CREATINE AS PERCENTAGE OF TOTAL CREATININE
<i>May</i>	<i>cc.</i>	<i>grams</i>	<i>grams</i>	<i>gram</i>	
25	1490	9.089	0.9622	0.2298	19.28
26	1360	8.704	1.0600	0.1416	11.78
27	1320	8.316	0.8450	0.1830	17.80
28	1265	10.626	0.9768	0.1668	14.58
29	1200	8.760	0.7840	0.1347	14.65
30	1390	9.174	0.7978	0.4218	34.59
31	1410	9.306	0.9486	0.3534	27.14

TABLE VI.

Pig, fed nitrogen, as rolled oats, equivalent to 38.5 times his creatinine nitrogen output. N intake, 7.28 grams.

DATE	VOLUME OF URINE	TOTAL N	CREATININE PRE-FORMED	TOTAL CREATININE
<i>August</i>	<i>cc.</i>	<i>grams</i>	<i>gram</i>	<i>gram</i>
9	1375	5.19	0.478	0.470
10	1940	6.92	0.532	0.532
11	1680	6.38	0.529	0.519
12	1380	7.17	0.555	0.555

In table VI is shown the behavior of a pig on a ration of rolled oats containing thirty-eight and a half times the nitrogen eliminated by the animal daily as creatinine. No appreciable amounts of creatine could be found in the urines.

It seems logical to conclude, from the data we have collected, that the plane of protein intake is not the controlling factor in regulating the creatine elimination in the growing pig. The character of the proteins in the diet appears to determine whether a large or small creatine production takes place in the tissues. *These observations leave little doubt that creatine may come from exogenous as well as endogenous protein metabolism.*

The observations of Mendel and Rose that feeding protein alone to fasting rabbits does not check the rate of creatine elimination also appears in a new light. We would explain this by assuming that, while the protein fed relieved the animal of rapid tissue destruction and creatine production (from endogenous sources), the exogenous protein catabolism set up simultaneously a creatine production which exceeded the animal's power to destroy, so creatine kept coming in the urine. This theory would necessitate the assumption, which our work with pigs leads us to believe would hold, that a fortunate selection of the protein fed would cause a cessation of creatine excretion in fasting rabbits. It is interesting to speculate as to the group in the protein molecule which is responsible for the formation of creatine. The only known cleavage product which suggests itself from similarity of constitution is arginine, which by a partial oxidation of the carbon chain to acetic acid and a methylation of a nitrogen atom would lead to creatine formation. This has been suggested by Jaffé¹³ and by Knoop¹⁴ and has some experimental evidence to support it, *viz.*, the methylation of guanidine acetic acid in the organism.¹⁵ From the hypothesis of Seemann¹⁶ that creatinine is not formed from arginine itself but from certain arginine peptides, it is possible that creatine may result from arginine only when the latter exists in certain combinations. If this were the case there could be no relationship between the arginine content of the proteins fed and the creatine production since only a part of the arginine might exist in complexes which could yield creatine in the body.

Our knowledge of the arginine content of naturally occurring food products is too meager to admit of conclusions regarding a possible relationship between the arginine content of the rations and the creatine content of the urines in our own experiments. Also the body doubtless destroys some creatine, a fact which probably accounts for the marked irregularities observed in the content of creatine in the urine of an animal on a uniform diet.

The working hypothesis that all arginine in the ration may contribute to creatine formation, would seem, in the light of our

¹³ Jaffé: *Zeitschr. f. physiol. Chem.*, xlviii, p. 430, 1906.

¹⁴ Knoop: *Zeitschr. f. physiol. Chem.*, lxvii, p. 495, 1910.

¹⁵ Dorner: *Zeitschr. f. physiol. Chem.*, lii, p. 246, 1907.

¹⁶ Seemann: *Zeitschr. f. Biol.*, xlix, p. 433, 1907.

observations, to be worth further examination, despite the negative result of Jaffé who failed to observe an increased elimination of creatine when arginine was subcutaneously injected into rabbits. The state of nutrition of the animal could easily turn the result of such an experiment in one way or the other. Folin¹⁷ has observed that neither creatine nor the corresponding nitrogen introduced into the body when the protein metabolism is at a very low level appears in the urine. His observation that creatine could be traced into the urine only when introduced with a high protein diet is also of interest in this connection.

We expect to continue a study of this problem.

¹⁷ Folin: *Hammarsten's Festschrift*, 1906.

SYNTHESIS OF LECITHIN IN THE HEN AND THE CHARACTER OF THE LECITHINS PRODUCED.¹

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One of the earliest proofs of the possession of synthetic power by the animal was the demonstration that fat can originate from carbohydrate in the animal body.² We now have indisputable evidence that the animal cell is capable of synthesizing nuclein and phosphatide complexes necessary for growth,³ and also that certain amino-acids, glycocoll and lysine and possibly also tryptophane, can originate in the animal body as the result of synthetic activity.⁴ Lusk has shown that carbohydrate can be derived from protein or from certain amino-acids.⁵ Up to the time of the experiments described in this paper, however, no very great production of lecithins and other phosphorus-containing lipoids had been shown to result from the synthetic activity of the animal cell.⁶ Since the experiments here described were completed, Fingerling⁷ has reported results which fully corroborate our own and which indicate that certain complex lipoids of the lecithin and kephalin

¹ Published with the permission of the Director of the Wisconsin Experiment Station.

² See Rosenfeld: *Ergeb. d. Physiol.*, i, p. 661, 1902.

³ McCollum: *Amer. Journ. of Physiol.*, xxv, p. 120, 1909.

⁴ Osborne and Mendel: Carnegie Institution of Washington, Publication 156, pts. 1 and 2, 1911; also McCollum: *Amer. Journ. of Physiol.*, xix, p. 215, 1911.

⁵ Lusk: *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

⁶ A brief statement of a part of the results reported in this paper was presented before the American Society of Biological Chemists at the Baltimore meeting, December, 1911. See Proceedings of the Society of Biological Chemists, this *Journal*, xi, p. xiii, 1912.

⁷ Fingerling: *Biochem. Zeitschr.*, xxxviii, p. 448, 1912.

types can be synthesized in large amounts by birds taking rations free from this class of compounds.

Experiments with fat-free rations compounded of isolated and purified food substances have not yet been successfully employed in experiments involving reproduction.⁸ In our own work we have employed a ration consisting of 30 per cent Merrill-Soule's skim milk powder and 70 per cent polished rice, the latter thoroughly extracted with boiling alcohol. The milk powder contained 0.6 per cent of ether-soluble matter and the ether extract was practically free from phosphorus. The lecithins present in the whole milk are nearly completely removed with the cream by the centrifugal process. The rice, before extraction, was ground to a fine powder. Two extractions were made, using a liberal amount of 95 per cent alcohol and maintaining a boiling temperature for from twenty to thirty minutes. It was then transferred to a press and nearly freed from alcohol, and finally spread in a thin layer until the alcohol was all evaporated. The rice thus treated was practically fat- and lecithin-free.

The hens selected for the experiment were hatched in June 1910, and were fed an ordinary mixed ration until October 31 when they were placed in a cage, the floor of which was covered with clean sand. The hens were kept in the attic of the poultry building throughout the experiment. There was no failure of the appetite at any time. The initial weights of the birds were as follows: No. 102, 967 grams; No. 103, 1476 grams; No. 106, 1184 grams.

The average consumption of food per day from October 31, 1910, to March 1, 1911, was 175.6 grams for the three or 58.5 grams per day per bird. The energy intake was about 235 calories per day for each hen. The weights of the hens on January 30, at which date the first eggs were secured, were as follows: No. 102, 1290 grams, gain 33 per cent; No. 103, 2013 grams, gain, 36.4 per cent; No. 106, 1588 grams, gain, 34.1 per cent.

The hens were not trap-nested so no individual records for egg production can be given. Between January 30 and April 15, fifty-seven eggs were secured. It is known that at least three eggs were eaten by the hens. The egg production was therefore not far from normal for pullets of this age.

⁸ See Osborne and Mendel: this *Journal*, xii, p. 81, 1912.

The yolks from the fifty-seven eggs weighed, as separated from the whites, 883.5 grams, an average yolk production of 294.5 grams per hen. The average white production per hen was 465.5 grams. As the eggs were collected they were weighed, broken and the whites and yolks separated and separately weighed. The yolks were dropped into 95 per cent alcohol and preserved until the end of the experiment.

A mixed sample of these yolks was found, by the method of Koch and Woods,⁹ to contain 3 per cent of lecithin and 6.39 per cent of kephalin, a total phosphatide content of 9.39 per cent. Each hen deposited in the egg-yolks produced by her 27.65 grams of phosphorized fats. It is fair to assume that the bodies of the hens contained more phosphatides at the end of the experiment than at the beginning, and also that some phosphatides were present in the whites. The fat content of the fresh yolks was 32.8 per cent.

Since the average daily consumption of skim milk powder was 17.55 grams, the fat content of which was 0.105 gram, the fat intake per hen during the feeding period of one hundred and sixty-five days was 17.37 grams. Being ordinary butter fat most of it was not suitable for direct deposition in the eggs. It is evident therefore that the synthesis of phosphatides is readily accomplished in the body of the hen when the ration is free from these substances.

We possess but little evidence as to whether the lecithins of the egg yolk are of a specific nature, or are of a variable character and influenced by the character of the fats or lecithins of the food. If the lecithins of the yolk are transferred without change to the nervous system of the developing chick it would seem highly probable that the fatty acid radicals of the lecithins must be of a specific and unvarying character. It is highly improbable that the composition of the nervous system of the chick, as respects the phosphatides, which form a part of the functioning organized matter, can vary through the substitution of, for example, oleyl-stearyllecithin, which might be abundant in the food, for oleyl-palmityllecithin, which the animal might synthesize if compelled to do so by lack of compounds of this character in the diet. On the other

⁹ Koch and Woods: this *Journal*, i, p. 203, 1905.

hand, that portion of the lecithin which is broken down during incubation to supply phosphoric acid for skeletal formation might, so far as one can see, be equally efficient if made of any of the ordinary fatty acid radicals, since these all yield about the same amount of energy. Mesernizky¹⁰ has shown that the lecithin content of the egg decreases markedly during incubation.

Bang¹¹ holds that since the proteins as we know them in the purified state are relatively stable substances, and we have no evidence that any high degree of lability exists in the proteins present in the living protoplasm, it is justifiable to attribute greater importance to the unstable complex lipoids which form a part of every living cell, than to the proteins; that the lipoids, among all the components of the colloidal systems which manifest the properties of living matter, are the most labile, and deserve therefore to be assigned an important rôle in the metabolic processes. If this assumption be justified one would expect to find for a portion of the lecithins of the yolk a specificity of composition and structure. This view is supported by the work of Henriques and Hansen¹² who conclude that the fatty acids of yolk lecithins are constant in character (iodine number) and independent of the character of the food lipoids, while the fats of the eggs are variable and easily influenced by the fats of the diet.

It should be stated that in the experiments of Henriques and Hansen the periods of experimental feeding were shorter than thirty days in six periods and were forty-two days in the other two, and eggs were collected during the entire periods. An effort was made to change by this method the nature of the fatty acid radicals of the egg lecithins by feeding barley, peas, rice, hempseed and linseed, and iodine numbers of 72.5, 70.8, 66.9, 74.7 and 78.4 respectively were obtained for the lecithins. There is no evidence that in their experiments a synthesis of lecithins actually occurred.

Of still greater interest is their observation that the iodine numbers of all lecithins examined by them were such as to indicate for the fatty acids themselves an iodine number of about 100, which would indicate the presence of acids having a higher iodine number than oleic acid (80-90). The idea of a specificity of the

¹⁰ Mesernizky: *Biochem. Centralbl.*, vi, p. 784, 1907.

¹¹ Bang: *Ergeb. d. Physiol.*, vi, p. 134, 1907.

¹² Henriques and Hansen: *Skand. Arch. f. Physiol.*, xiv, p. 390, 1903.

character of the lecithins is strengthened by their observation that preparations of lecithins from dog and ox brains showed nearly the same iodine numbers (96-100) as did those from egg lecithins.

These results of Henriques and Hansen make especially interesting the iodine numbers of lecithins produced by our hens where a complete synthesis of all the organic complexes of the lecithins was made absolutely certain. The following table gives the values for the fats and lecithins of the eggs produced in the experiments here described, and, for comparison, the corresponding values for eggs from the farm flock on an ordinary ration.

	IODINE NUMBER		
	I	II	III
<i>Fats</i>			
Nearly lipid-free ration..	50.0	54.36	51.1
Ordinary ration.....	63.2	65.5	
<i>Lecithins</i>			
Nearly lipid-free ration..	35.22	34.07	34.0
Ordinary ration.....	63.7	63.1	

The method of obtaining the lecithins was as follows:

The yolks were removed from the alcohol and placed in an evaporating dish, covered with a watch glass and heated on a steam bath for a short time to remove most of the alcohol. Ether was then added repeatedly in small portions and the evaporation continued until the alcohol was all removed. By this process the yolks were protected as much as possible from the air. The yolks were then dried in a desiccator over sulphuric acid, the desiccator being evacuated by the ether method. When dry, the sample was transferred to an extraction apparatus and thoroughly extracted with ether. The ether was evaporated, the last part being removed over sulphuric acid in a desiccator. Great care was taken to prevent exposure of the fat and lecithin preparations to the air. The residue from the ether extraction was extracted with seven or eight portions of hot alcohol (95 per cent), each digestion being continued about fifteen minutes. The combined alcohol extracts were concentrated on a steam bath. Toward the end of the evaporation small portions of ether were repeatedly added to hasten the removal of the alcohol and to protect the lipoids from the air. The product was finally dissolved in ether and precipitated with ten volumes of acetone. After once reprecipitating, the lecithins were dried in a vacuum desiccator over sulphuric acid. The alcohol in which the eggs were preserved was evaporated in the manner described and the residue treated in the same way as the yolks.

The iodine numbers were made on different samples and there is no doubt that they represent the true values for the fats and lecithins from the mixed eggs. From these results we must conclude that the lecithins of the egg yolk are variable in respect to the nature of the fatty acid radicals they contain and that their nature is influenced by the character of the lipoids of the diet. The iodine numbers of lecithins in yolks from a nearly lipoid-free diet correspond fairly well with those which one might expect for a lecithin containing one molecule of oleic and one of a saturated fatty acid. It is significant that the lowest iodine number obtained by Henriques and Hansen was from a rice diet which was very poor in lipoids.

Unfortunately none of the eggs with these low iodine numbers were hatched, but this will be done as soon as more can be produced. It will be of interest to compare the lecithins of the nervous system of chicks, hatched from such eggs, with those of chicks obtained after feeding rations similar to those of Henriques and Hansen, when the rations are fed long enough to secure the maximum influence of an abundance of highly unsaturated lipoids of the diet on the lecithins of the egg.

METABOLISM STUDIES ON COLD-BLOODED ANIMALS. I.

THE URINE OF THE FISH.

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Probably on account of the difficulties attendant on their collection the data at hand regarding the composition of the urine of marine animals are extremely limited, and decidedly fragmentary in character; in fact so far as the author is able to ascertain, no analyses showing the distribution of nitrogen in twenty-four-hour quantities of urine in even the larger marine animals, such as the fish, are to be found in the literature.

As, by means of the micro-chemical methods for the analysis of urine recently published by Folin and his associates, it is now possible to make observations on the distribution of nitrogen in twenty-four-hour samples of the urine of relatively small animals, the time seemed ripe for an effort to investigate the metabolism of lower animals, from the standpoint of urine analysis, more thoroughly than has so far been attempted.

As the great majority of fish possess no bladder but allow the urine to escape as soon as formed, it was first necessary to find some method by which this fluid could be collected accurately over twenty-four-hour periods. My original intention was to study the urine of both the elasmobranchs and of the teleosts; it was found impossible, however, in the period during which the work of collection was carried on to obtain enough specimens of any one species of bony fish to make work along this line profitable; in the present paper, therefore, the data presented consist largely of observations on the urine of one of the elasmobranchs, the smooth dog fish (*Mustelis canis*), which is extraordinarily abundant along the north Atlantic coast. Analytical data concerning the urine of

one bony fish, the goose fish (*Lophius piscatorius*), are also presented, the material for this work having been taken from the bladder shortly after death.

The method used for the collection of twenty-four-hour samples of urine from the dog fish was as follows.¹

Large male animals were used almost entirely. The female may be employed but in the male the insertion of the cannula is accomplished with much greater ease than in the female. The animal was first securely fastened, dorsal surface up, in a holder consisting of a V-shaped trough about 18 inches long and 6 inches deep. The fish fastened to the holder was then placed in a long narrow tank of running sea water in such a position that while the head including the gills was under water the remaining two-thirds of the body including the cloaca was above the surface of the liquid.

The cloaca was then stretched open by means of hooks inserted laterally and an ordinary glass cannula inserted and securely tied directly into the urinary papilla. The latter operation is particularly easy to accomplish in the mature male in which the papilla may be as much as a quarter of an inch in length. In the male the cannula may now be tied by means of thread to the "claspers." In the female it is necessary to suture it to the fins in order that it may not be pulled out during the subsequent struggles of the fish.

The animal was now removed from its holder, placed on its side and carefully tied by means of inch wide bandages to a board about 2 feet in length. The whole was now placed in a large tank, well supplied with running sea water, and was fastened in such a position that the cloaca was raised above the surface of the liquid. A wet towel placed over the tail kept the latter moist.

The urine was collected in a large test tube which was hung on the side of the tank considerably below the head of the fish. This test tube was closed by means of a two-hole rubber stopper, through one hole of which passed a short glass tube, which in turn connected with a piece of narrow pure gum tubing, the other end of which was attached to the cannula in the urinary papilla.

All urine samples were preserved with chloroform and were analyzed for nitrogenous constituents not later than thirty hours after collection.

The method outlined above is extremely simple and has the advantage that as no surgical procedure is necessary no anaesthetics need be used and at the same time elimination from the intestine is not interfered with. Gentle pressure applied in the

¹ The method outlined above for the collection of urine was suggested to me by Mr. Scott of the Department of Natural History of the College of the City of New York. I have also to thank Mr. Scott for much valuable assistance during the development of the method.

median line of the dorsal surface about 1 to 6 inches above the cloaca will serve to expel any urine present in the ureters and, if this procedure be employed just before insertion and just previous to removal of the cannula, a fairly accurate measure of the amount of urine excreted in a given period of time may be obtained.

If it is not desired to keep the fish bound to a board during the collection it may be conveniently placed in a trough so narrow that the animal is unable to turn around; I have used this method in a few cases but lack of conveniently arranged tanks made its exclusive adoption impossible.

The method described above differs fundamentally from the procedures used by Herter,² by Baglioni³ and by Burian⁴ for the collection of fish urine in the fact that I have always arranged the cannula so that the urine was drained away as soon as formed. The quantity of urine eliminated in twenty-four hours by the dog fish is surprisingly small considering the size of the animal, as will be shown by the results tabulated below. All animals used were males.

FISH NO.	LENGTH	WEIGHT	VOLUME OF URINE COLLECTED IN TWENTY- FOUR HOURS
	cm.	grams	cc.
26	94	2268	15
29	76	1134	11
30	86	1814	27
31	71	964	17
32	92	2041	30
35	69	964	25
36	81	1588	22
37	81	1360	27

The dog fish survives pithing of the cord remarkably well and will usually live apparently in good condition for several days after the operation. As in many physiological experiments involving the collection of urine destruction of the cord is to be desired, I have made a number of collections of twenty-four-hour quantities of urine from fish in which the cord had been destroyed up to about the level of the dorsal fin.

² Herter: *Mitth. aus der zool. Stat. zu Neapel*, x, p. 342, 1891.

³ Burian: *Zeitschr. f. biol. Technik u. Method*, i, p. 383, 1909.

⁴ Baglioni: *Zentralbl. f. Physiol.*, xix, p. 385, 1903.

FISH NO.	SEX	LENGTH	WEIGHT	VOLUME OF URINE COLLECTED IN TWENTY-FOUR HOURS
		cm.	grams.	cc.
1	♀	92	1815	20.0
3	♀	94	2722	21.0
9	♂	81	1446	19.5
11	♀	96	1810	13.0
18	♀	96	1588	20.0
19	♂	107	2948	44.0
20	♂	79	1174	17.0
21	♂	91	1813	17.5

As will be seen by an inspection of these results the quantity of urine eliminated in twenty-four hours is about the same in the pithed as in the normal animal, the average twenty-four-hour quantity for the former being 21.5 cc. and for the latter, 21.7 cc. An examination of the analytical results given below will show, however, that in the pithed animal a much less concentrated urine is secreted than in the normal fish.

When first passed, the urine of the dog fish is a clear odorless, almost colorless fluid with a slight yellowish-green tinge and a reaction distinctly acid to litmus. After standing for a few days, even when preserved with chloroform, it becomes cloudy, and the color darkens gradually until when several weeks old it possesses the color of a moderately concentrated human urine.

In all, the urine of about thirty animals was examined and in no case was any reducing sugar of albumin found. The murexide reaction is readily obtained. Creatinine appears to be present, but the amount is so small that quantitative measurements were impossible, even by the Folin method. No creatine could be detected.

The analytical methods used were as follows: specific gravity by the hydrometer, total nitrogen by the method of Folin and Farmer,⁵ urea by the potassium acetate method of Folin,⁶ ammonia and uric acid by the methods of Folin and Macallum,⁷ phosphates

⁵ This *Journal*, xi, p. 493, 1912.

⁶ *Ibid.*, xi, p. 507, 1912.

⁷ *Ibid.*, xi, pp. 265, 523, 1912. (The method used for the colorimetric determination of uric acid will be published shortly from this laboratory.)

by the uranium acetate titration and total sulphur by the author's³ modification of the Benedict method. The animals from which urine was collected had in every case been caught from twenty-four to forty-eight hours before the experiment was started, and during this time were kept in cages immersed in the ocean.

Fish 30.

Male; weight, 1814 grams; fasting; volume of urine collected in twenty-four hours, 27 cc.; specific gravity, 1.032.

	MILLIGRAMS IN TWENTY-FOUR HOURS	MILLIGRAMS PER CC.	PER CENT OF TOTAL NITROGEN
Total nitrogen.....	122.5	4.5	
Urea nitrogen.....	108.4	4.0	89.0
Ammonia nitrogen.....	2.3	0.085	1.9
Uric acid nitrogen.....	0.31	0.011	0.25
Creatinine nitrogen.....	Present		

Fish 29.

Male; weight, 1134 grams; fasting; volume of urine collected in twenty-four hours, 11 cc.

	MILLIGRAMS IN TWENTY-FOUR HOURS	MILLIGRAMS PER CC.	PER CENT OF TOTAL NITROGEN
Total nitrogen.....	58.3	5.3	
Urea nitrogen.....	51.5	4.7	88.6
Ammonia nitrogen.....	1.32	0.12	2.2

Fish 31.

Male; weight, 964 grams; volume of urine collected in twenty-four hours, 17 cc. This animal was allowed to feed on a large amount of fish muscle about six hours before the collection of urine was commenced.

	MILLIGRAMS IN TWENTY-FOUR HOURS	MILLIGRAMS PER CC.	PER CENT OF TOTAL NITROGEN
Total nitrogen.....	113.0	6.6	
Urea nitrogen.....	93.4	5.5	82.6
Ammonia nitrogen.....	5.18	0.30	4.5

³ This *Journal*, viii, p. 401, 1910.

Urine of the Fish

Fish 19.

Male; weight, 2948 grams; fasting; spinal cord pithed from tip of tail to about the level of the dorsal fin; volume of urine collected in twenty-four hours, 44 cc.

	MILLIGRAMS IN TWENTY-FOUR HOURS	MILLIGRAMS PER CC.	PER CENT OF TOTAL NITROGEN
Total nitrogen.....	104.7	2.37	
Urea nitrogen.....	88.2	2.00	84.2
Ammonia nitrogen.....	3.3	0.075	3.1
Creatinine nitrogen.....	Present		
Creatine nitrogen.....	None		
Uric acid nitrogen.....	0.56	0.012	0.5

Fish 19a.

Male; weight, 2940 grams; fasting; spinal cord pithed; volume of urine collected in twenty-four hours, 27 cc.

	MILLIGRAMS IN TWENTY-FOUR HOURS	MILLIGRAMS PER CC.	PER CENT OF TOTAL NITROGEN
Total nitrogen.....	62.00	2.3	
Urea nitrogen.....	53.00	1.96	85.2
Ammonia nitrogen.....	3.78	0.14	6.1
Uric acid nitrogen.....	0.40	0.015	0.64
Creatinine.....	Present		

Mixed urines of fishes 20 and 21, both males; weight of fish 20, 1174 grams; volume of urine for twenty-four hours, 17 cc.; weight of fish 21, 1813 grams; volume of urine for twenty-four hours, 17 cc.; both fasting; spinal cord pithed in both.

	MILLIGRAMS PER 1000 CC.	PER CENT OF TOTAL NITROGEN
Total nitrogen.....	3200	
Urea nitrogen.....	2600	81.0
Ammonia nitrogen.....	340	10.6
Creatinine nitrogen.....	Present	
Creatine nitrogen.....	None	
Uric acid nitrogen.....	22	0.68

Analysis of a composite sample of urine collected from ten fasting dog fish.

Specific gravity, 1.030.

	GRAMS PER LITER	PER CENT OF TOTAL NITROGEN
Total nitrogen.....	4.200	
Urea nitrogen.....	3.390	80.7
Ammonia nitrogen.....	0.310	7.3
Chlorides (as NaCl).....	12.86	
Phosphates (as P_2O_5).....	4.520	
Total sulphur (as SO_4).....	7.08	
Total sulphates (as SO_4).....	3.40	

The goose fish (*Lophius piscatorius*) proved of all the teleosts available to be the best suited for the study of the urinary secretion, as this animal is provided with a bladder of large capacity. However, during the time spent at Woods Hole only one specimen was available in which the bladder contained urine. In this case 158 cc. of urine were removed from the bladder about an hour after death and immediately analyzed. The urine of the goose fish is a clear, odorless, almost colorless fluid, which after long standing (when preserved with chloroform) becomes faintly yellow. The specific gravity was found to be 1.013. The reaction is distinctly acid to litmus. No albumin or reducing sugar could be detected. As in the urine of the dog fish the amount of phosphates is so large that when the urine is boiled a thick flocculent precipitate is obtained which readily dissolves on the addition of a drop of acetic acid. On making alkaline with sodium hydrate a large amount of precipitate is also obtained probably due to the presence of an excess of earthy phosphate.

Uric acid is apparently not present as the murexide test gave negative results, while even with the very delicate phosphotungstic acid reagent⁹ (which gives unmistakable results with 1 part of uric acid in 500,000 parts of water), used in the colorimetric method for the quantitative determination of the substance, no indication of its presence could be found.¹⁰

⁹ Folin and Denis: this *Journal*, xii, p. 237, 1912.

¹⁰ This is in agreement with the observations of Rywosch on the urine of the carp: *Wien. med. Wochenschr.*, 1893, pp. 47, 48; and with the earlier work of Davy: *Trans. Roy. Soc. Edinburgh*, xxi, p. 543, 857.

No creatine or creatinine could be detected by the use of the Jaffé test.

	MILLIGRAMS PER LITER	PER CENT OF TOTAL NITROGEN
Total nitrogen.....	400	
Urea nitrogen.....	248	62
Ammonia nitrogen.....	52	13

It is planned to extend this investigation to a study of the metabolism of a number of the cold-blooded animals, such as the fish, and of such of the amphibia and reptiles as may be found available for this type of work.

MAINTENANCE EXPERIMENTS WITH ISOLATED PROTEINS.¹

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In earlier papers on our feeding experiments with isolated food substances² we have attempted to justify the selection of the white rat for the study of some of the problems connected with nutrition. This animal is easily reared and cared for. Its small size reduces the food requirement to a magnitude which falls within the range of experimental possibility where the preparation of special dietaries by laborious processes is a fundamental prerequisite. A possible advantage in the use of smaller animals like those which we have selected lies in the fact that marked changes in nutritive equilibrium speedily manifest themselves. Furthermore, the longevity of this animal is, according to Donaldson, about three years; so that the first year of life corresponds to a long span in terms of human years. Not insignificant is the additional fact that the white rat has in recent years been made the subject of exceptionally extensive measurements in respect to growth and various features of development at the Wistar Institute in Philadelphia and elsewhere. In this way physical standards, so to speak, have been established for this animal. Inasmuch as we have successfully maintained albino rats over periods of more

¹ The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

² Osborne and Mendel: Feeding Experiments with Isolated Food Substances. Carnegie Institution of Washington, Publication 156, Parts I and II, 1911; *Zeitschr. f. physiol. Chem.*, lxxx, p. 307, 1912; this *Journal*, xii, p. 473, 1912.

than 600 days on artificially prepared food mixtures we believe that the adaptability of the animal for the purposes under consideration must be admitted. The chief obstacles to success have been these diseases, usually involving the lungs, which other workers with rats have also found troublesome. These diseases, however, have made less inroad upon our carefully selected and cared for experimental animals than upon the animals of our stock colony. We believe that by the application of further hygienic precaution the disease factor can be largely eliminated.

The work of earlier investigators, who have attempted to determine whether any single protein or combination of proteins is capable of supplying the nitrogenous needs of the body, has been reviewed in an earlier publication.² If we except the investigations of Röhmman,³ the details of which have not yet been published, it would appear from the records of our predecessors that "experiments in which one form of protein has been given as the sole source of nitrogen for a long period demonstrate that, in spite of the abundance of nitrogen in the diet, the animal ceases to thrive."⁴ Experiments in which the foodstuffs administered were sufficiently "pure" to give some permanent significance to the results obtained are few in number. Occasional records show that animals have been kept over one hundred days; as a rule, however, part of this period has been characterized by a decline in body weight which obviously must vitiate the success of any demonstration of maintenance on "artificial" diets. The reserve store of nutrients in many animals is sufficient to keep them alive for considerable periods even on inadequate dietaries, so that the continuance of life is by no means equivalent to adequate nutritive maintenance. This fact has too often been overlooked in the interpretation of nutrition experiments.

The futility of studying nutrition by methods in which even the "control" animals decline ought to be obvious and is brought to mind by recent experiments of Frank and Schittenhelm⁵ who failed

² Osborne and Mendel: Carnegie Institution of Washington, Publication 156, Part I, p. 6, 1911; see also Cathcart: *The Physiology of Protein Metabolism*, 1912, p. 74.

³ Röhmman: *Biochem. Zeitschr.*, xxxix, p. 507, 1912.

⁴ Cathcart: *The Physiology of Protein Metabolism*, 1912, p. 74.

⁵ Frank and Schittenhelm: *Therapeutische Monatshfte*, xxvi, p. 112, 1912.

to nourish rats adequately on a diet of egg-white, starch, glucose, fat, cellulose and salt mixture. Their rats declined within thirty days on this diet which was selected to be compared with a dietary in which completely digested proteins formed the source of nitrogen. The authors emphasize the fact that such digestion products may at times contain objectionable toxic substances (amines?). Their insistence on the use of "pure" proteins, however, loses part of its significance in the light of the fact that none of their artificial feedings were reasonably successful even with supposedly favorable selections of diet.

In explanation of the failures of our predecessors various suggestions have been offered. The failure to eat sufficient food has clearly been a frequent obstacle, as has been emphasized among others by McCollum.⁷ Aside from this, however, "much of the earlier work in this connection," as Cathcart remarks, "was faulty owing either to the manner in which the experiments were carried out, or to the fact that the diets could not be regarded as 'pure,' i.e., the protein used was not absolutely free from impurities." He adds: "Notwithstanding this it has been found that if the animals be kept for a prolonged period on one diet they invariably die in spite of an abundant caloric intake."⁸ We propose to consider this criticism carefully in the experiments to be reported below.

The necessity of long continued experiments calls for particular emphasis. Physiological alterations dependent upon the gradual depletion of a small store of essential tissue material may manifest themselves with extreme slowness; and the fact that a satisfactory nutritive balance can be maintained for a week or two or even a month in some cases is no guarantee of either the ultimate success of the dietary or of the impossibility of a decline owing to the inappropriate exhibition of an essential ingredient (cf. Charts 1, 2 and 3). This has further been brought out most strikingly in the splendid study of Hart, McCollum, Steenbock and Humphrey on the physiological effects on growth and reproduction of rations balanced from restricted sources.⁹ They have shown that animals fed rations from different plant sources and comparably balanced

⁷ McCollum: *Amer. Journ. of Physiol.*, xxv, p. 120, 1909.

⁸ Cathcart: *The Physiology of Protein Metabolism*, 1912, p. 74.

⁹ Hart, McCollum, Steenbock and Humphrey: University of Wisconsin Agricultural Experiment Station, Research Bulletin No. 17, 1911.

in regard to the supply of digestible organic nutrients and production therms were not alike in respect to general vigor, size and strength of offspring and capacity for milk secretion. The records extend over three years so that the gradual manifestations of departure from the normal standards of health and physiological efficiency which failed to reveal themselves in the earlier period were not overlooked. As these authors say, "unquestionably the physiological value of a ration is largely dependent upon its chemical constituents, but the usual determinations made on feeding materials do not reveal the character or manner of combination of many of the constituents. Consequently the physiological value can be determined, in the present state of our knowledge, only by long continued observations of the reaction of the feed on the animal."

A consideration of the prolonged maintenance of any animal raises the question as to its natural duration of life. In the case of the white rat we know of few statements which can lay claim to any accurate basis. The current view, based apparently on a statement by Donaldson, that the average life span of the albino rat is about three years, rests on the fact that there had been no records of a survival of this period in animals under observation. Recent studies by Slonaker¹⁰ indicate that a white rat can live to an age of 1361 days. The incidence of disease in a large rat colony must be taken into account in connection with duration tests; and it has proved to be less detrimental among our isolated experimental animals than in the larger colony where the animals are maintained on mixed food under conditions more nearly like those to which they are accustomed by nature. In fact the mortality among our experimental rats has thus far been smaller than among our supply animals.

The weight of the animals constitutes, we believe, the best index at the present time of satisfactory nutritive maintenance. The necessity of distinguishing carefully between nutrition in maintenance and in growth has been emphasized in another place.¹¹

¹⁰ Slonaker: *Journal of Animal Behavior*, ii, pp. 20-42, 1912; also *The Effect of a Strictly Vegetable Diet on the Spontaneous Activity, the Rate of Growth, and the Longevity of the Albino Rat*, Leland Stanford Junior University Publications, 1912.

¹¹ Osborne and Mendel: *Zeitschr. f. physiol. Chem.*, lxxx, p. 307, 1912.

Perhaps, as Waters has suggested, the term *maintenance* has been used somewhat loosely in the past; but like others we have been in the habit of regarding the animal in maintenance when its live weight was constant. "A more correct definition of the term would perhaps be to say that an animal was in maintenance when its body was in energy balance, but the live weight has been the conventional measure of our maintenance values."¹² In the present report we are concerned solely with the maintenance features.

Under selected conditions of diet an animal can be maintained adequately without growth (cf. Chart 4). Furthermore a reparation of tissue is not necessarily identical with growth (cf. the weight gained by Rat 134, in Chart 5, days 72 to 120, which shows that it is possible to restore tissue loss, exemplified in decline of body weight, by the use of dietaries which are inadequate for growth). Recovery from the decline due to malnutrition, for example, may thus be brought about by gliadin feeding. The possible dissimilarity of the processes of maintenance, growth, and repair, in so far as they affect the rôle of proteins in nutrition, has also been emphasized by McCollum.¹³ The distinctions here made are illustrated by many of the charts in the appendix.

It is perhaps unnecessary to remark that there may be involved in the problems of maintenance and growth many other factors, viz., the total energy intake, the character of the inorganic salts, the specific nature of the carbohydrates in the diet, quantitative and qualitative differences¹⁴ in the proteins administered as well as the indefinable so-called "hormones." Indeed some writers at the present time believe that in the latter, as yet unknown factors, rests the secret to nutritive success. Thus Cathcart has lately written, "it is clear that apart from the caloric intake and the protein, carbohydrate and fat content of the food, there is some factor or factors which influences the utilization, perhaps

¹² Waters: The Capacity of Animals to grow under Adverse Conditions, *Proceedings Society for the Promotion of Agricultural Science*, xxix, p. 3, 1908.

¹³ McCollum: *Amer. Journ. of Physiol.*, xxix, p. 215, 1911.

¹⁴ The choice of a protein content of about 18 per cent in our food mixtures has been determined by nutrition trials with varying concentrations of protein in the diet. These will be described elsewhere. The proportions here selected fall within the range of our most successful experiments, when the non-protein factors are otherwise the same.

also the amount of food required. This evidence practically points to the presence of some 'mineral' substance, or substances, in normal food which are absent in 'pure' food.¹³ In an earlier report it was stated that an animal had been fed more than 217 days on a diet in which the sole protein was glutenin. The complete record of this animal, Rat 71, is given in Chart 6, since it illustrates a number of important features connected with our feeding experiments. It will be noted that the decline after 300 days of experimental feeding which could not be prevented by the addition of a second protein, edestin, to the diet was speedily prevented by the administration of mixed food during a period of one week. When the earlier glutenin food was again fed in period 8 a gradual decline again set in; this was however promptly averted, and striking reparation made, when "protein-free milk" was used to replace the inorganic salts and part of the carbohydrates of the dietary. On this food mixture the animal continued for 144 days until death ensued after 531 days of experimental feeding, the immediate cause being an abscess of the jaw resulting in the inability of the animal to eat. Old age may have contributed materially to the final decline of this rat. A peculiar significance centers about period 6 on the curve because it clearly shows that the factors determining successful nutrition were here involved in some other component of the dietary than the protein itself. The reasons which have led to the use of the protein-free milk as an adjuvant of our dietaries, as well as the possible criticisms in respect to a minimal protein content which may attach to its use, have been presented in detail elsewhere.¹⁴

We have already called attention to the necessity of long continued experiments if the actual sufficiency of a diet is to be determined. A deficiency in some essential ingredient may not make itself manifest for a long time, and even then be difficult to detect; for many other causes than defects in the food may lead to the decline or death of animals during experiments lasting over many months. It is only when the failure to be maintained is

¹³ Cathcart: *The Physiology of Protein Metabolism*, 1912, p. 76; cf. also Hopkins: *Journ. of Physiol.*, xlv, p. 425, 1912; Suzuki, Shimamura and Odake: *Biochem. Zeitschr.*, xliii, p. 89, 1912.

¹⁴ Osborne and Mendel: Carnegie Institution of Washington, Publication 156, Part II, 1911; *Zeitschr. f. physiol. Chem.*, lxxx, p. 307, 1912; *This Journal*, xii, p. 483, 1912.

the invariable outcome of the prolonged feeding on a given diet that we are justified in assuming that the diet is in some way inadequate, and then only when a prompt and complete recovery ensues when the diet is changed to one that is known to be in all respects sufficient (cf. Chart 1, period 3; Chart 2, period 4; Chart 3, period 5; and Chart 6, period 6). Our experience shows that every animal has sooner or later declined when fed with mixtures of isolated and purified proteins, carbohydrates and fats together with inorganic matter in the form of crystallized salts. In nearly every case the decline has been sudden, with strong evidence that death would soon have ensued had not the food been changed. In each case immediate recovery has followed a change in the diet, thus showing the experimental foods to be inadequate for prolonged nutrition.

Whether the deficiency of the purely artificial diet is to be attributed to improper proportions of its constituents, to improper combinations of these constituents, or to the lack of some essential element, is at present difficult to determine. That the elements essential for prolonged maintenance are present in milk from which the fat and protein have been removed is also shown in the charts already referred to. In every case the substitution of a food containing its inorganic constituents and a part of its carbohydrate in the form of this so-called "protein-free milk" has resulted in immediate recovery of the depleted animal, and thereafter the animal has continued in a well-nourished state until its life terminated from disease or old age. If we compare the body weight curves of mature animals maintained in nutritive equilibrium on the purely artificial diet with those of animals fed on a diet known to be deficient in some element supposed to be essential for maintenance, we find a marked difference. Thus animals kept on a diet free from inorganic salts or on one containing as its sole protein either zein or gelatin, which lack tryptophane, immediately decline and continue to fall in weight throughout the entire time of such feeding; whereas in the experiments with the purely artificial mixtures containing adequate proteins and inorganic salts a decline in weight occurs only after some time, and is then, in nearly every case, sudden and severe. That no serious damage has been done to the animal is shown by its rapid recovery when protein-free milk is added to the food; however, that it has suffered in some way is indicated by the fact that when restored to its

original weight it cannot then be again maintained for any considerable time when returned to the original diet.

Whether or not this means that its cells have lost something essential for their normal activity, which is furnished by the "protein-free milk," cannot now be determined; but the experimental evidence is sufficient to justify a search for such a substance in milk. Recently published investigations by Hopkins¹⁷ indicate that milk, as well as other natural food materials, contains a substance or substances which, even in very small quantities, suffices to induce normal and continued growth, for several weeks at least, in rats maintained on artificial mixtures of food substances which are otherwise inadequate for growth.¹⁸

Charts 5, 7, 8, 9, 10 and 11 show that with the aid of "protein-free milk" it is possible to maintain rats for periods equal to practically their entire adult lives on foods containing a single purified protein, and also that the successful food proteins may differ very widely in their chemical make-up without affecting the physical well being of the animal to any noticeable extent. If we consider that after hydrolysis with acids gliadin yields 25 per cent of its nitrogen as ammonia, while casein and edestin yield only 10 per cent, and that gliadin yields only 5.8 per cent of its nitrogen as basic amino-acids while casein yields 22.2 per cent and edestin 31.4 per cent, it is evident that the form in which the nitrogen is presented to the animal is very different for each of these proteins. These differences are further emphasized by the proportion of some of the amino-acids determined by methods which give results of sufficient accuracy to justify comparison.

	CASEIN	EDESTIN	GLIADIN
Arginine.....	3.81	14.17	3.16
Histidine.....	2.50	2.19	1.56
Lysine.....	5.95	1.65	*
Glutaminic acid.....	15.55	18.74	43.66
Glycocoll.....	0.00	3.80	0.00

* Regarding the presence of lysine in gliadin, see Osborne and Mendel: *This Journal*, xii, p. 480, 1912.

¹⁷ Hopkins: *Journ. of Physiol.*, xliv, p. 425, 1912.

¹⁸ Cf. also Suzuki, Shimamura and Odake: *Biochem. Zeitschr.*, xliii, p. 89, 1912.

Another important difference, which has usually been overlooked when comparing casein with other proteins, is that only about 0.1 per cent of sulphur can be obtained as sulphide from casein, whereas from edestin 0.35 and from gliadin 0.62 per cent can be obtained, thus showing that casein contains very little cystine.

The possibility of successful maintenance is not by any means restricted to the particular proteins employed in these experiments. It happens, however, that our longest records involve those proteins which earliest elicited our experimental interest, and which could be prepared in pure form most advantageously.

Special importance centers in the remarkably successful maintenance of rats upon gliadin. The continuance of Rat 130, for example (see Chart 11), during more than 530 days of adult life on a mixture of isolated food substances containing a single protein deficient in two familiar Bausteine, lysine and glycocoll,¹⁹ and which affords an inadequate diet for satisfactory growth, furnishes by far the longest experiment on record of "artificial" nutrition and should serve to justify the renewal of studies with the isolated foodstuffs. We have at present records of twelve rats which have been maintained more than 400 days on comparable food mixtures and five animals whose maintenance record exceeds 500 days. These prolonged nutrition trials exceed in duration the best maintenance records which we have thus far obtained in our stock colony of animals fed on mixed foods. Bearing in mind that these diets are, in addition to their probable freedom from glycocoll in the case of casein, and their deficiency in glycocoll and lysine in the case of gliadin, devoid of more than the merest possible traces of purines and of phosphoproteins, except in the case of casein, the synthetic activities of the organism are again clearly brought to mind. It would surely be an extreme exaggeration of every metabolic probability to assume that in periods extending far longer than a year, i.e., approximately one-half of an animal's span of life, the organism had conserved its store of the missing chemical complexes or altered its chemical make-up. The latter assumption indeed is completely at variance both with the available evidence regarding the chemical

¹⁹ Regarding the possible presence of lysine and glycocoll in gliadin, see Osborne and Mendel: *This Journal*, xii, p. 480, 1912.

fixity of the tissues and biological considerations respecting the identity of species.

The possible criticism which may be evoked by the use of "protein-free milk" containing a minute trace of unremoved milk protein (equivalent to about 0.5 per cent of the entire food), can be met satisfactorily only by maintenance experiments which we are now conducting with artificial mixtures of inorganic salts and carbohydrates.²⁰ The trials have as yet continued over too short periods to be in any way comparable with the longer records above. A few charts are, however, appended (see Charts 12 and 13) because they already indicate a considerable degree of success in the absence of milk protein as well as of the hypothetical organic "hormones," etc., present in the milk. The experiences with the artificial salt mixture I (see Charts 2, 7, 10 and 14) afford evidence on this point, for in these experiments the animals were maintained in good health for 190, 360, 400 and 200 days respectively before declining in weight.

As a corollary, so to speak, to the preceding experiments in successful maintenance are appended a few records of failures of maintenance associated with the administration of manifestly inadequate proteins (cf. Charts 15, 16, 17, 18, 19, 20 and 21). In several of these the ready restoration of body weight by the addition of, or replacement by, adequate protein is clearly indicated. It is interesting to note the more or less successful repair of nutritive failure by gliadin (see Charts 17, 18 and 21) which is inadequate to promote true growth.²¹

The failure to be maintained on the inadequate proteins, zein and gelatin, is not to be ascribed to a failure in utilization; for nitrogen determinations made on the feces showed that the utilization of the protein was good.

DISCUSSION.

The currently discussed theories of metabolism emphasize the importance of the "Bausteine" as fundamental factors in protein metabolism; and they lay stress by inference, if not by experiment,

²⁰ Osborne and Mendel; *Proc. Soc. for Exp. Biol. and Med.*, ix, p. 72, 1912.

²¹ The physiological rôle of gliadin is discussed by Osborne and Mendel: *This Journal*, xii, p. 473, 1912.

upon the results of supplying these in adequate proportions as a primary requisite.²² Now and then there has arisen a protest against the general view that the similarity of the molecule of the food protein to that of the specific body proteins determines its relative food value to the animal, and that any one of the essential cleavage products which is present in smallest amount in the food protein determines the value of the entire molecule to the animal. McCollum,²³ for example, has presented experimental data which do not harmonize entirely with the most widely accepted theories concerning the chemistry of protein metabolism and which he interprets to indicate that the processes of cellular catabolism and repair do not involve the construction and resynthesis of the entire protein molecule. Abderhalden has lately summarized his view of the situation in these words:

Hier müssen wir allerdings eine zurzeit noch grosse Lücke in unseren Kenntnissen besonders hervorheben. Wir wissen noch sehr wenig über die Fähigkeiten der tierischen Zellen Aminosäuren zu bilden. Nur für Glykokoll ist bewiesen, dass es aufgebaut werden kann. Ferner wissen wir von Tryptophan, dass es offenbar von den Körperzellen nicht gebildet wird. Ebenso scheinen die aromatischen Bausteine nicht ersetzbar zu sein. . . . Es muss somit der Möglichkeit gerechnet werden, dass die Zelle imstande ist, manchen Baustein zu ergänzen und damit das zu Gebote stehende Aminosäuregemisch besser verwertbar zu machen. Durch die Hervorhebung dieser Lücken in unseren Kenntnissen über den intermediären Zellstoffwechsel wird das Hypothetische in unseren Vorstellungen über den Ablauf des Eiweissstoffwechsels im tierischen Organismus mit Absicht besonders betont.²⁴

The experimental records presented in the preceding pages suggest that we have in the past greatly underestimated the possibility of a transmutation or synthesis of amino-acids in the organism, and that these chemical processes may play a more significant part in nutrition than has been credited to them hitherto. The realization of the possibility of a transmutation of amino-acids has an obvious bearing upon the question of the quantity of protein in the diet; for the arguments in favor of a liberal protein

²² Cf. Mendel: *Ergb. d. Physiol.*, xi, p. 418, 1911; Cathcart: *The Physiology of Protein Metabolism*, 1912.

²³ McCollum: *Amer. Journ. of Physiol.*, xxix, p. 215, 1911.

²⁴ Abderhalden: *Synthese der Zellbausteine in Pflanze und Tier*, Berlin, 1912.

intake have in part been based upon the belief that all of the Bausteine must be supplied in adequate amounts. The ability to be maintained over long periods on the chemically unique protein gliadin is an excellent illustration of the point raised.

To what extent the daily "wear and tear" of metabolism may require a new supply of Bausteine to replace depleted tissue, remains a matter of conjecture at present. Studies to determine the amount of nitrogenous material involved in what Rubner has termed the "Abnutzungsquote" show that it is not large in quantity. The experiments here recorded give no answer to the possible extent of the synthesis of new amino-acids except perhaps in the case of glycocoll and lysine. Further investigations with the various proteins must be undertaken to determine the *relative minimum quantities* of each of them essential for long continued maintenance. Our foods ordinarily contained 18 per cent of protein—a proportion of the total intake found in the case of casein to be larger than the necessary minimum of about 6-7 per cent of the food. Similar experiments are now in progress with gliadin and edestin.

That a protein as unlike the tissue proteins as is gliadin can, in fact, serve for the construction of new tissues through the intervention of the metabolic processes of the mature animal is no longer to be doubted in view of an experiment reported in another paper,²⁵ in which a pair of rats maintained 178 days on gliadin as the sole protein in the diet produced four healthy young and successfully reared them. This involved not only the construction of the tissues of the young, but also the production of the milk by which they were successfully nourished.

The recent observation of Grafe and Schläpfer on the protein-sparing action of organic ammonium salts,²⁶ as well as those of Abderhalden,²⁷ taken in connection with the striking experiments of Knoop and Embden on the synthesis of individual amino-acids under purely experimental conditions, brings the synthetic powers of the animal organism into new prominence. Indeed as Grafe remarks:

²⁵ Osborne and Mendel: *This Journal*, xii, p. 473, 1912.

²⁶ Grafe and Schläpfer: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 1, 1912; Grafe: *ibid.*, lxxviii, p. 485, 1912.

²⁷ Abderhalden: *ibid.*, lxxviii, p. 1, 1912.

Da wir hier erst am Anfange unserer Kenntnisse auf einem ganz neuen Gebiete des intermediären Stoffwechsels und der synthetischen Leistungsfähigkeit des tierischen Organismus stehen, haben zunächst natürlich alle Deutungsversuche nur sehr untergeordnete Bedeutung und können nur als Arbeitshypothesen dienlich sein.

Further evidence of the physiological efficiency of animals maintained on single proteins is brought out by the fact that the capacity of reproduction has not been impaired in these animals and no obvious physical defects or unusual behavior were discernible.

The long records from the continued use of unchanged rations afford further confirmation of what we have earlier pointed out, namely, that monotony of diet is not necessarily a troublesome factor and is not of such importance in nutrition problems as is usually supposed.²⁸

To what extent alimentary bacteria may intervene to furnish adequate building stones to the organism by utilizing the products of digestion found in the alimentary tract as their own nutritive pabulum and reconverting it by means of their eminent synthetic capacity into products suitable for the higher organisms cannot be definitely stated. It is not impossible that in the case of such substances as ammonium salts they may render some effective service by converting the nitrogen into other assimilable forms. We do not at present regard these bacterial possibilities as an adequate explanation of the nutritive success recorded; otherwise there is no apparent reason why a protein defective for growth should not be rendered efficient through this means. The contributions of the microorganisms to nutrient efficiency need further investigation and the possibilities have lately been clearly summarized by Armsby.²⁹

We believe that experiments such as those reported in this paper show the possibility of approaching certain of the problems of nutrition by new and hitherto discredited methods of study. The exceptionally long periods of feeding and observation involved in our successful trials are in striking contrast with most of the previously published records. If we had been content to discon-

²⁸ Cf. Hart, McCollum, Steenbock and Humphrey: University of Wisconsin, Agricultural Experiment Station, Research Bulletin No. 17, 1911.

²⁹ Armsby: The Nutritive Value of the Non-protein of Feeding Stuffs, Bureau of Animal Industry, Bulletin 139, 1911.

tinue the experiments after a reasonable period many of the declines evidently associated with imperfections in the dietary, and readily checked by a change in feeding, would have escaped attention. Such facts deserve to be considered in relation to current work in which the criteria of adequate nutrition on unusual diets such as amino-acid mixtures, etc., are sought in the nitrogen balance of the animals. None of the records, so far as we are aware, extend over periods even half as long as some of ours which ultimately ended in nutritive failure. Nitrogen balances may at times prove singularly deceptive, and give apparently favorable indications of equilibrium although the real nutritive status of the animal may be less promising. This is brought out in the consideration of some of the animals reported in an earlier publication.³⁰

Now that an animal can be maintained for long periods on a single protein there is prospect of a successful consideration of those other factors in nutrition which are even more illusive. The relative significance of the inorganic ions, and the qualitative value of the fats and carbohydrates which supplement the diet ought to be rendered amenable to study.³¹ In the case of the carbohydrates we have already made observations which suggest that it is no longer justifiable to consider these foodstuffs from a single standpoint without reference to their structural individuality. The consideration of such facts must, however, be reserved for other communications.

³⁰ Osborne and Mendel: Carnegie Institution of Washington, Publication 156, Part I, 1911.

³¹ Cf. Osborne and Mendel: *This Journal*, xii, p. 81, 1912.

APPENDIX.

Explanation of the Charts.

The abscissae of the curves represent days and the ordinates, actual body weight (solid line) or food-intake (dotted line) in grams. In some of the charts the average (normal) curve of growth, plotted from body weight data available for normally growing animals of the same sex, is represented by a broken line for comparison. The food intake curve is plotted from the quantities of food eaten per week. The numbers on the body weight curves indicate the time at which changes in the character of the feeding were instituted.

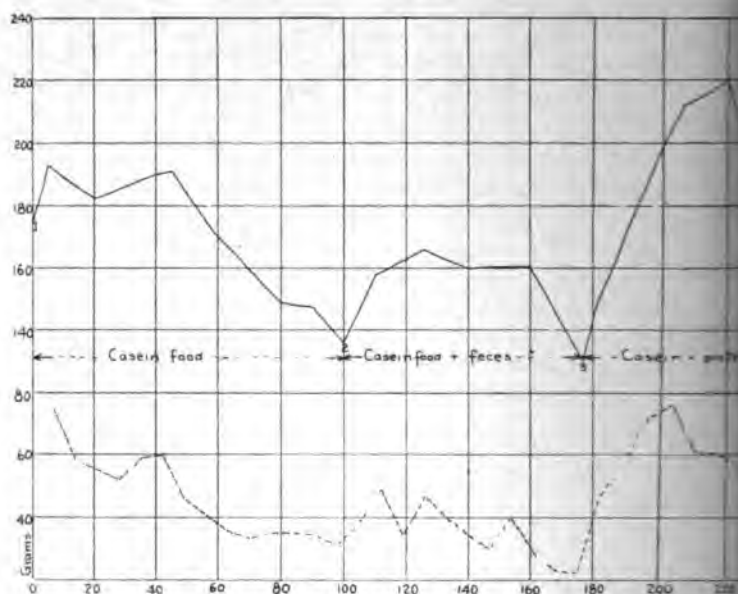
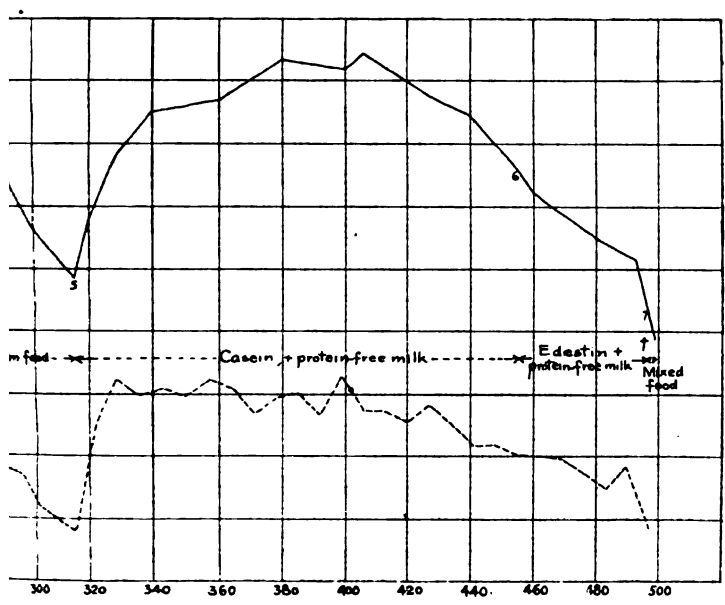


CHART 1, RAT 127 ♂, shows long-continued maintenance on the diet. The diet was given each week. The possible effect of this is discussed in the text. The rat was killed by diseased lungs after 499 days of experimental feeding.

PERIODS 1, 2 AND 4.

	per cent.	
Casein (cow's milk).....	18.0	Casein (cow's milk)
Starch.....	32.5	Protein-free milk
Sucrose.....	21.9	Starch.....
Salt mixture I.....	2.6	Agar.....
Lard.....	25.0	Lard.....
	<hr/> 100.0	



During period 2 about 1 gram of air-dry feces from rats on a mixed
61, Carnegie Institution of Washington. The animal's life was ter-

PERIOD 6.		
per cent.		per cent.
18.0	Edestin (hempseed).....	18.0
28.2	Protein-free milk.....	28.0
23.8	Starch.....	26.0
5.0	Lard.....	28.0
25.0		
100.0		100.0

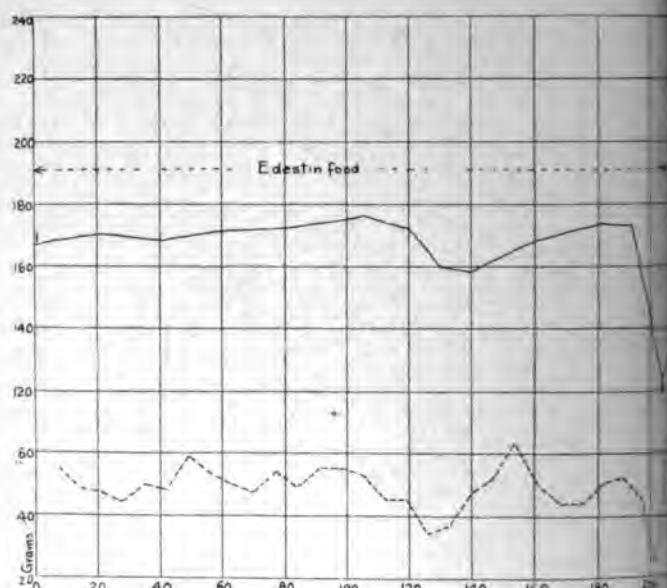
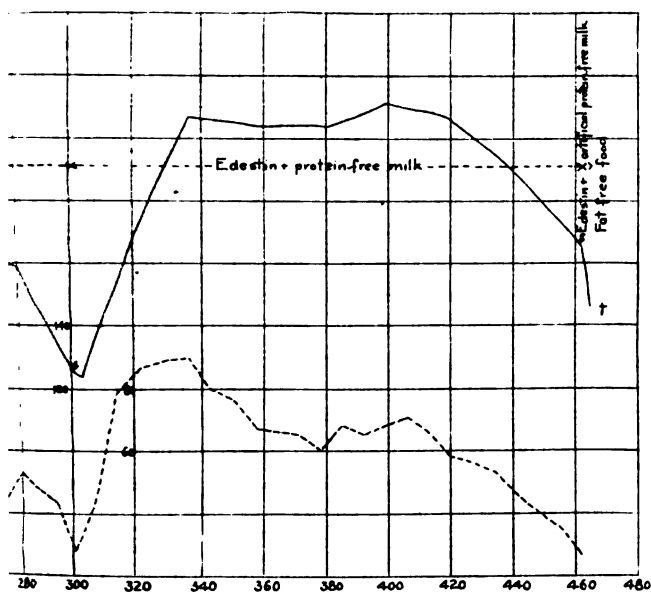


CHART 2, RAT 133 ♀, shows maintenance during 465 days on a diet of Edestin, organic salts and a part of the carbohydrate of the original food mixture. At the end of this time the animal's life was terminated by a tumor of the spleen.

The diet during the different periods is shown below.

PERIODS 1 AND 3.

	per cent.	
Edestin (hemp seed).....	18.0	Edestin (hemp seed).....
Starch.....	29.5	Protein-free milk.....
Sucrose.....	17.0	Starch.....
Agar.....	5.0	Agar.....
Salt mixture I.....	2.5	Lard.....
Lard.....	28.0	
	100.0	



from hempeed formed the sole protein. At the end of that
 very in periods 2 and 4 when protein-free milk replaced the inor-

PERIOD 5.

per cent.		per cent.
18.0	Edestin (hempeed).....	22.0
28.2	Artificial protein-free milk.....	29.5
20.8	Starch.....	28.5
5.0	Sucrose.....	20.0
28.0		
100.0		100.0

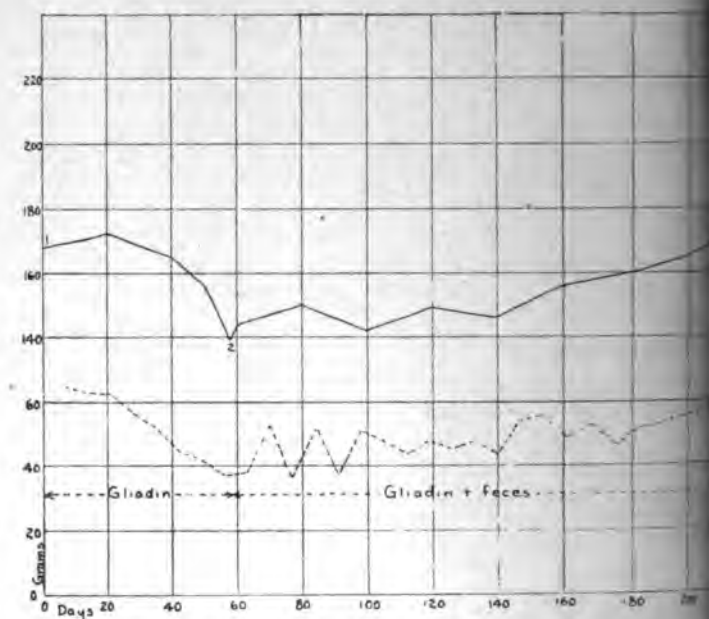
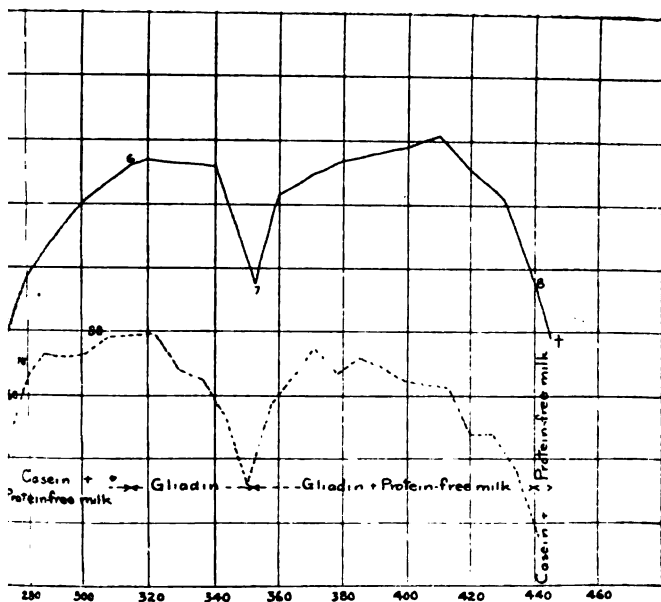


CHART 3, RAT 147 ♀, shows long continued maintenance on a diet of isolated proteins and starches after 445 days of experimental feeding.

The diet during the different periods is shown below. During period 1, see Chart 1.

PERIODS 1, 2, 3 AND 6.

	per cent.	
Glutadin (wheat)	18.0	Glutadin (wheat)
Starch	29.5	Protein-free milk
Sucrose	15.0	Starch
Agar	5.0	Agar
Salt mixture I	2.5	Lard
Lard	30.0	
	100.0	



as the sole protein. The animal's life was terminated by diseased
 mity of feces from rats on a mixed diet was supplied. See legend

PERIODS 5 AND 8.

per cent.		per cent.
18.0	Casein (cow's milk).....	18.0
28.2	Protein-free milk.....	28.2
20.8	Starch.....	23.8
5.0	Agar.....	5.0
28.0	Lard.....	25.0
100.0		100.0

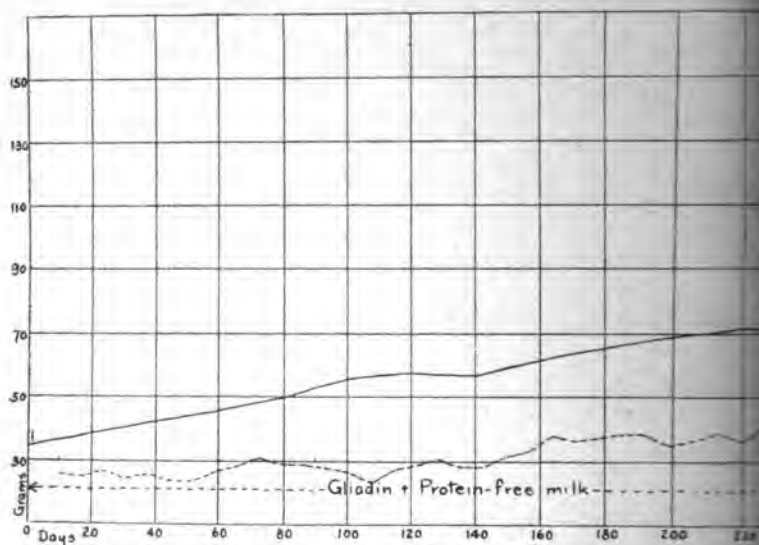
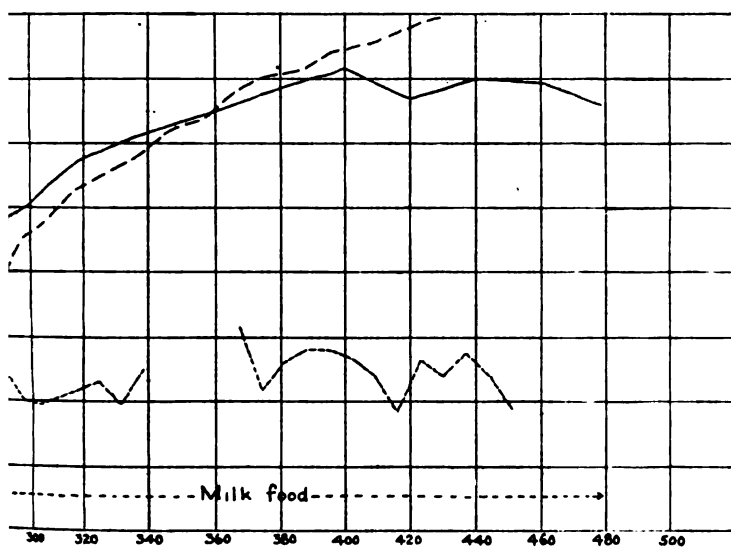


CHART 4, RAT 240 ♀, shows failure to make more than slight growth normal rate after 276 days of stunting. At this time the rat was 314. The diet during periods 1 and 2 was:

PERIOD 1.

Gladiin (wheat).....	
Protein-free milk.....	
Starch.....	
Agar.....	
Lard.....	



ning gliadin as the sole protein, and capacity to resume growth at a which rats normally grow very little more.

PERIOD 2.

	per cent.
.....	60.0
.....	16.0
.....	24.0
	<hr/>
	100.0

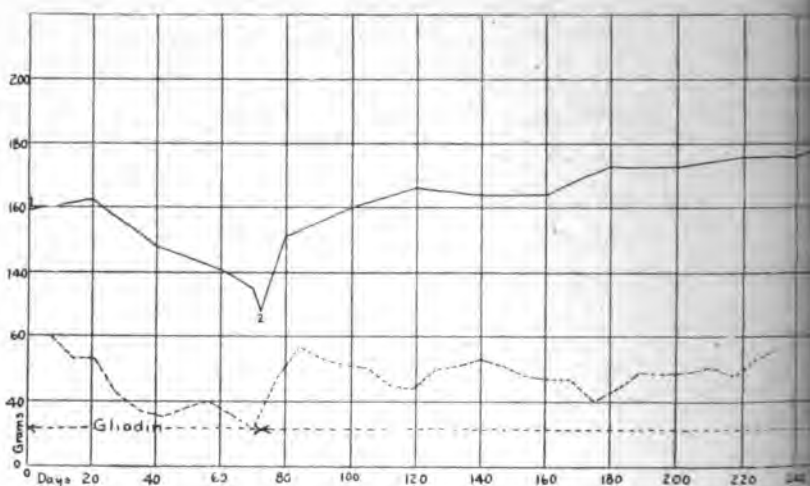
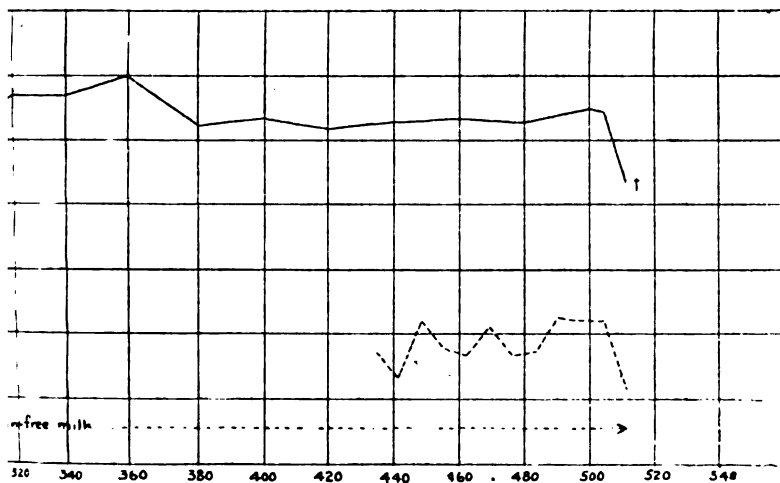


CHART 5, RAT 134 ♀, shows long continued maintenance on a diet containing mental feeding by an ulcer of the pylorus.

The diet during periods 1 and 2 was:

PERIOD 1.

Glutadin (wheat)
Starch
Sucrose
Agar
Salt mixture I
Lard



sole protein. The animal's life was terminated after 511 days of experi-

PERIOD 2.

	per cent.
fat)	18.0
milk	28.2
.....	20.8
.....	5.0
.....	28.0
	<hr/>
	100.0

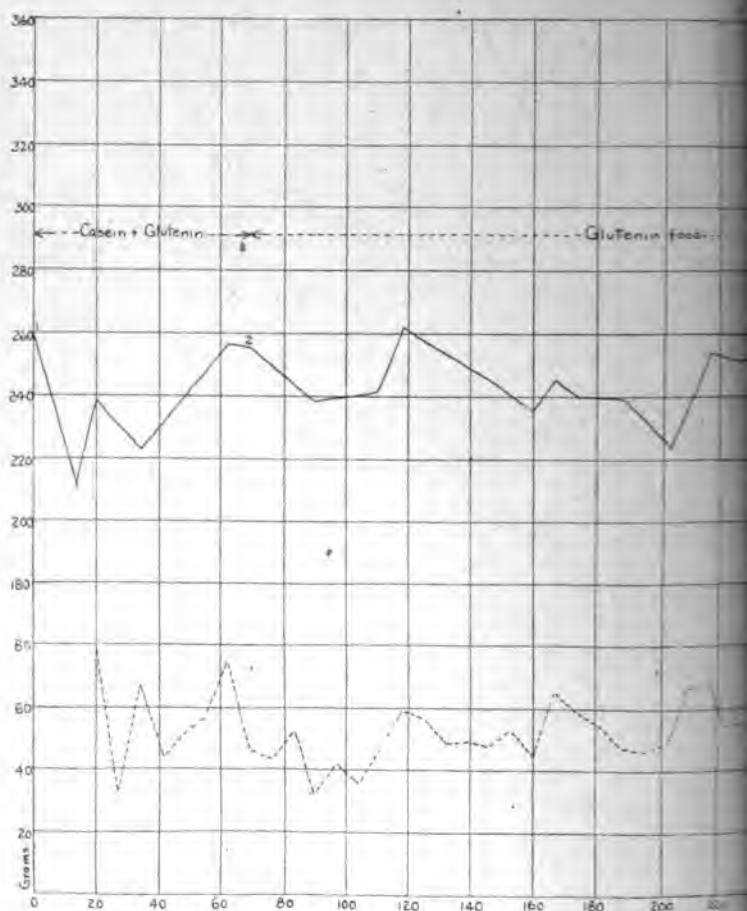
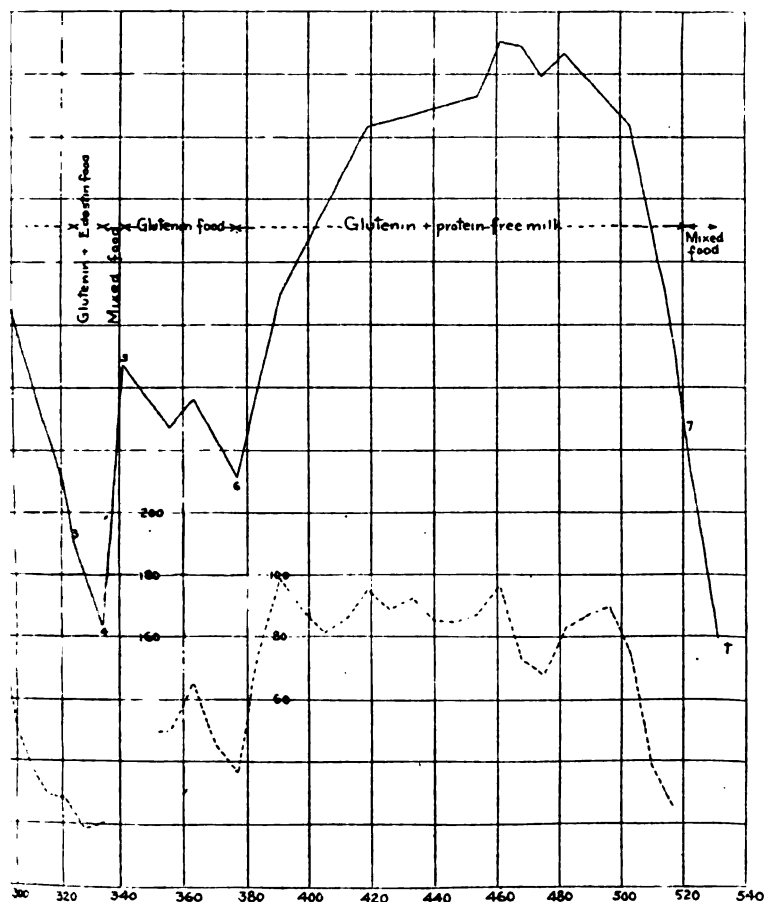


CHART 6, RAT 71 ♂, shows long-continued feeding of isolated foodstuffs. The rat's life was terminated after 531 days of experimental feeding by an abscess. The diet during the different periods was as follows:

PERIOD 1.		PERIODS 2 AND 5.	
	per cent.		
Glutenin (wheat).....	8.0	Glutenin (wheat).....	
Casein (cow's milk).....	12.0	Starch.....	
Starch.....	24.5	Sucrose.....	
Sucrose.....	15.0	Agar.....	
Agar.....	5.0	Salt mixture I.....	
Salt mixture I.....	2.5	Lard.....	
Lard.....	35.0		
	<hr/> 100.0		



tinued maintenance on glutenin from wheat as the only protein. The animal made eating impossible.

PERIOD 3.

per cent.

min (wheat).....	9.0
lin (hemp seed)	9.0
h.....	23.5
ss.....	18.5
.....	5.0
mixture I.....	2.5
.....	22.5
	<hr/>
	100.0

PERIODS 4 AND 7.

Mixed

PERIOD 6.

per cent.

Glutenin (wheat).....	18.0
Protein-free milk.....	28.2
Starch.....	23.8
Agar.....	5.0
Lard.....	25.0
	<hr/>
	100.0

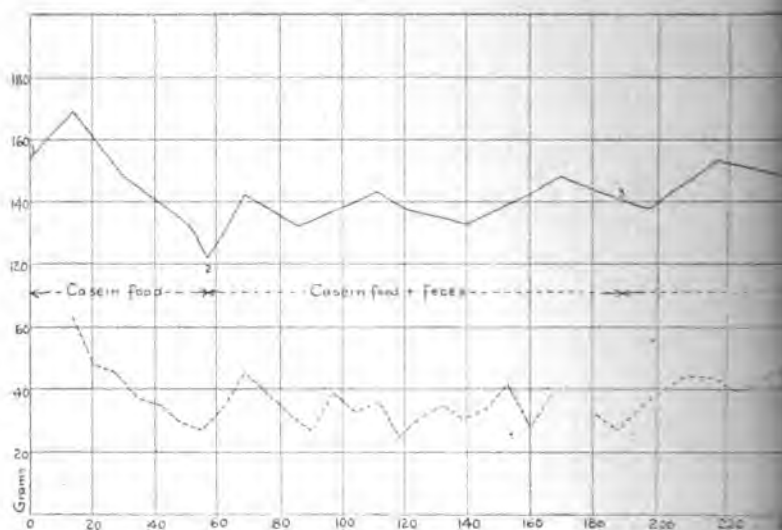
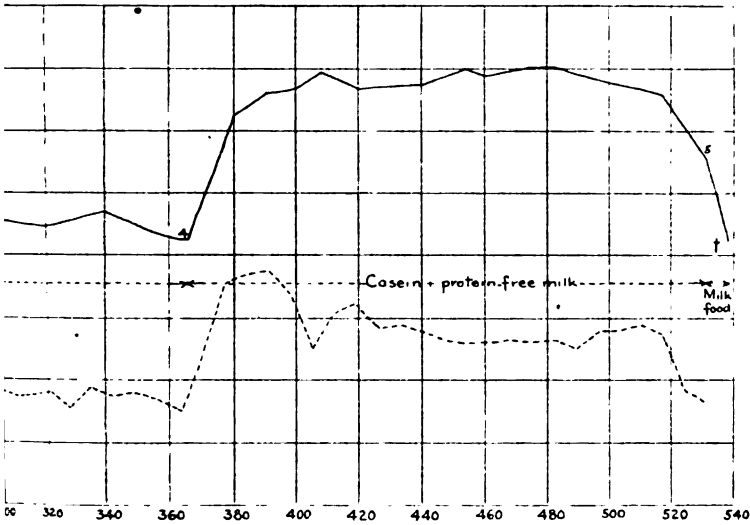


CHART 7, RAT 150 ♀, shows long-continued maintenance on a diet containing 18.0 per cent. casein for 230 days of experimental feeding.

The diet during the different periods is shown below. During period 2 a

PERIODS 1, 2 AND 3.

	per cent.	
Casein (cow's milk).....	18.0	Casein (cow's milk).....
Starch.....	32.5	Protein-free milk.....
Sucrose.....	21.9	Starch.....
Salt mixture I.....	2.6	Lard.....
Lard.....	25.0	
	100.0	



sole protein. The animal's life was terminated by diseased lungs after 538

fees from rats on a mixed diet was supplied. See legend on Chart 1.

PERIOD 5.			
per cent.			per cent.
18.0	Milk powder.....	60.0	
28.0	Starch.....	12.0	
27.0	Lard.....	28.0	
27.0			
100.0		100.0	

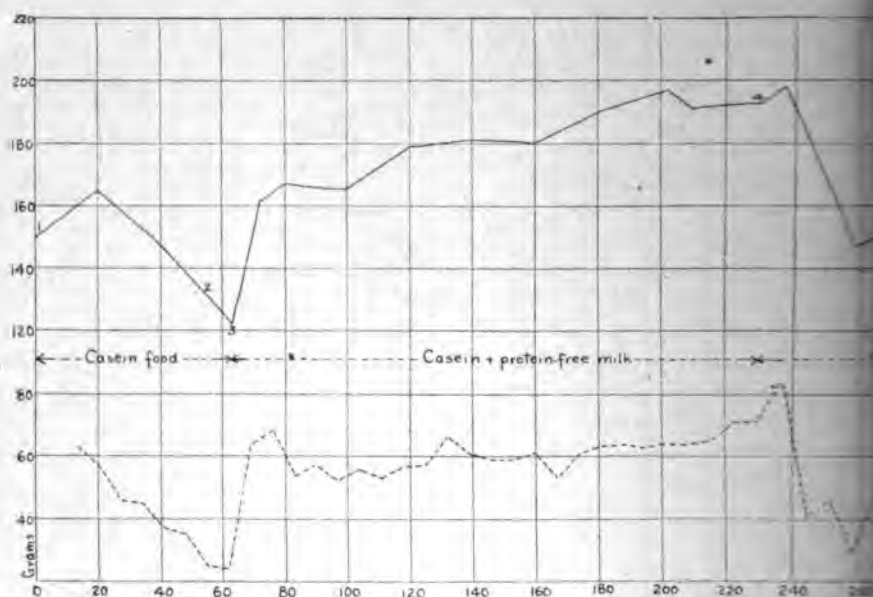
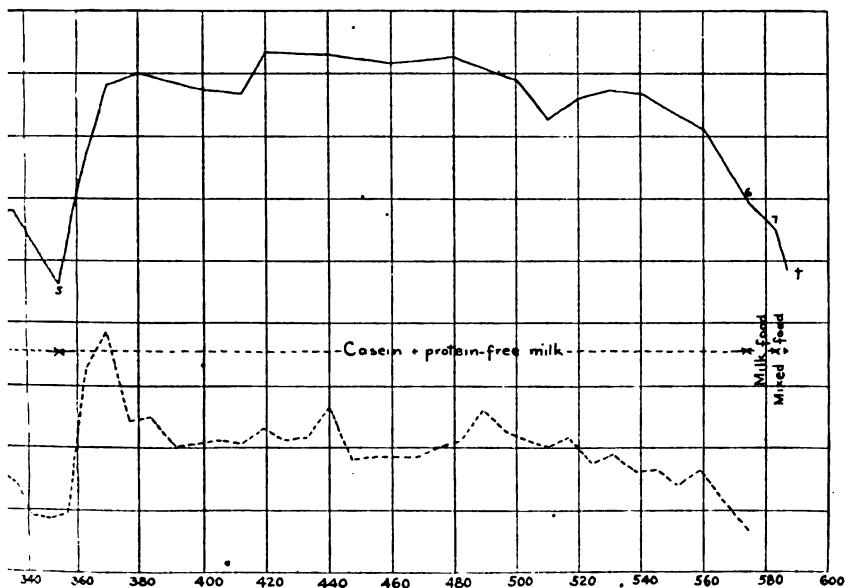


CHART 8, RAT 141 ♀, shows long-continued maintenance on a diet containing diseased lungs.

The diet during the different periods is shown below.

PERIODS 1 AND 4.			PERIOD 2.	
		per cent.		
Casein (cow's milk).....	18.0		Casein (cow's milk).....	
Starch.....	32.5		Starch.....	
Sucrose.....	21.9		Sucrose.....	
Salt mixture I.....	2.6		Salt mixture I.....	
Lard.....	25.0		Lard.....	
		100.0		



ein. After 587 days of experimental feeding the animal's life was terminated by

PERIODS 3 AND 5.

	per cent.
w's milk).....	18.0
* milk.....	28.0
.....	27.0
.....	27.0
	<hr/> 100.0

PERIOD 6.

	per cent.
Milk powder.....	60.0
Starch.....	12.0
Lard.....	28.0
	<hr/> 100.0

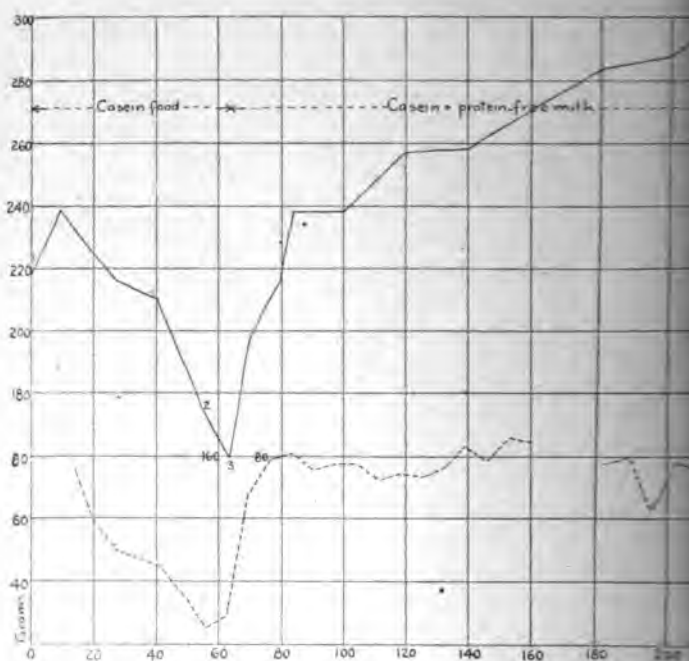
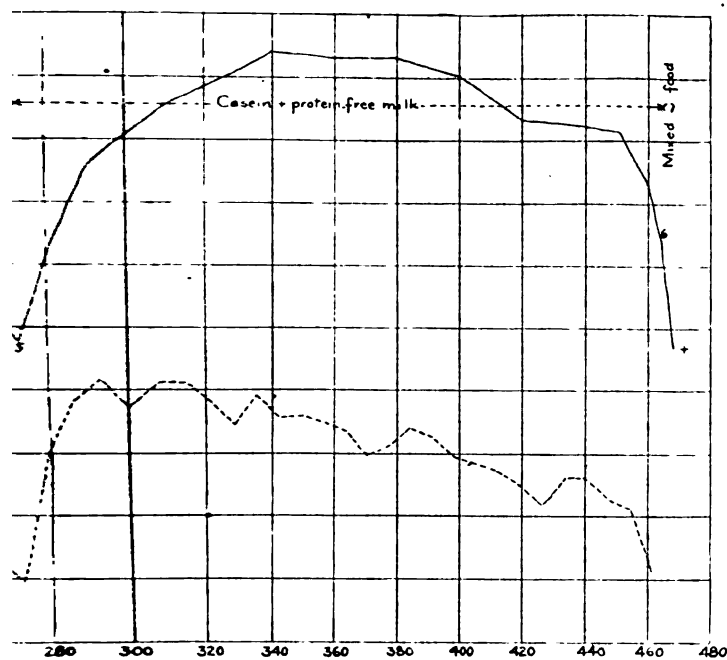


CHART 9, RAT 139 ♂, shows long-continued maintenance on a $\frac{1}{2}$ mental feeding, but no cause for death was shown by the autopsy. The diet during the different periods is given below.

PERIODS 1 AND 4.

	per cent.	
Casein (cow's milk).....	18.0	Casein (cow's milk).....
Starch.....	32.5	Starch.....
Sucrose.....	21.9	Sucrose.....
Salt mixture I.....	2.6	Salt mixture I.....
Lard.....	25.0	Lard.....
	100.0	



in as its sole protein. The animal died after 468 days of experi-

PERIODS 3 AND 5.

per cent.

36.0

22.5

13.9

2.6

25.0

100.0

Casein (cow's milk)..... 18.0

Protein-free milk..... 28.0

Starch..... 27.0

Lard..... 27.0

100.0

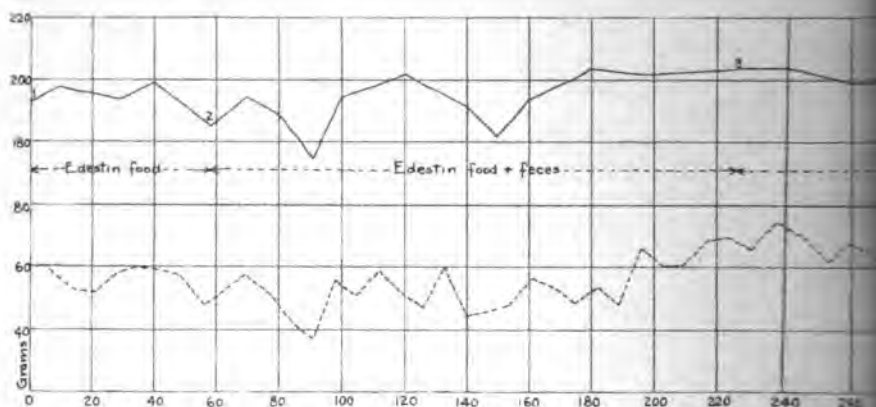
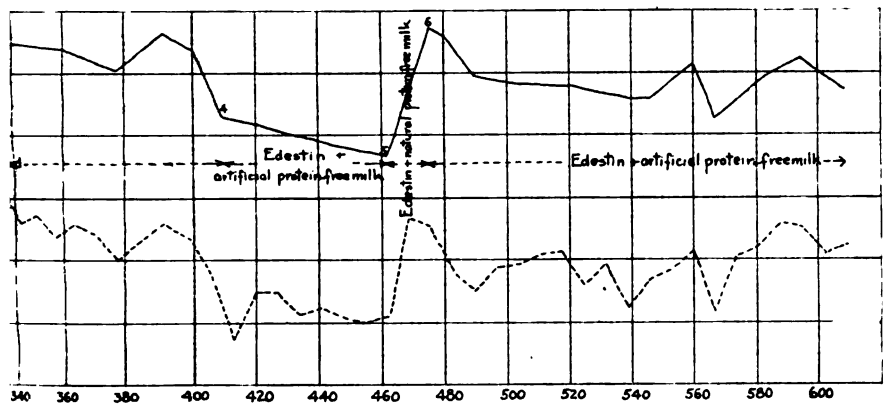


CHART 10, RAT 124 ♀, shows long-continued maintenance on a diet containing edestin 609 days of experimental feeding (which is, so far as we are aware, the longest maintenance on original weight. During period 2 the rat received small quantities of feces from nest

PERIODS 1, 2 AND 3.

	per cent.	
Edestin (hemp seed).....	18.0	Edestin (hemp seed).....
Starch.....	29.5	Artificial protein.....
Sucrose.....	17.0	Starch.....
Agar.....	5.0	Lard.....
Salt mixture I.....	2.5	
Lard.....	28.0	
	100.0	



2. Note that this rat has had natural protein-free milk for only 14 days out of the entire (obtained) and also that at the end of this period the animal is somewhat above its legend on Chart 1. This rat is still at its original weight after 637 days.

PERIOD 5.

per cent.		per cent
18.0	Edestin (hempseed).....	18.0
29.5	Natural protein-free milk.....	28.0
24.5	Starch.....	26.0
28.0	Lard.....	28.0
100.0		100.0

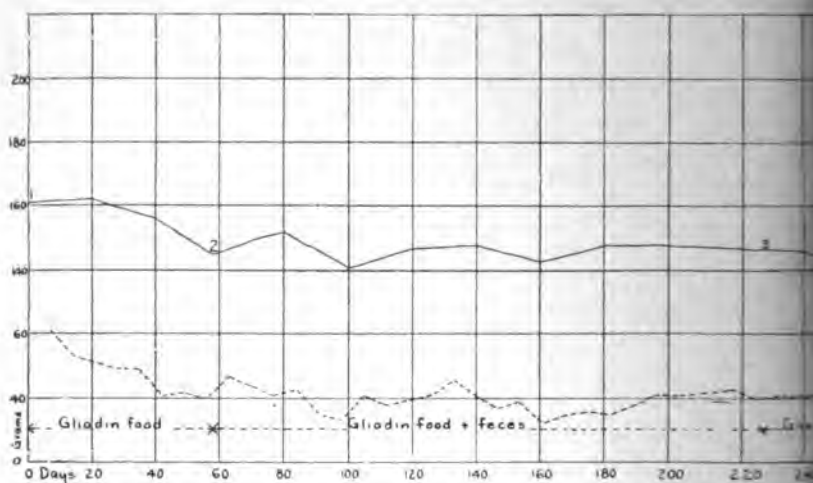
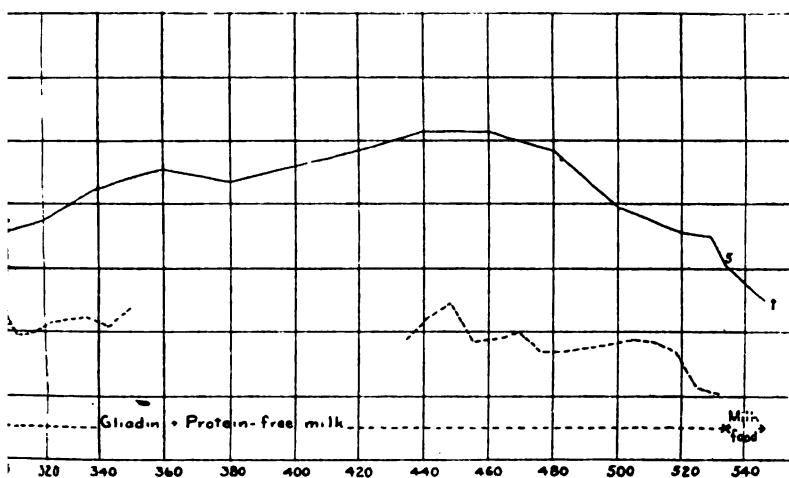


CHART 11, RAT 130 ♀, shows long continued maintenance on a diet ~~ment~~ mental feeding by diseased lungs and a large parasite, over 40 cm. long, cm. The diet during the different periods is shown below. During period 2

PERIODS 1, 2 AND 3.

	per cent.	
Gladiol (wheat).....	18.0	Gladiol (wheat).....
Starch.....	29.5	Protein-free salt.....
Sucrose.....	17.0	Starch.....
Agar.....	5.0	Agar.....
Salt mixture I.....	2.5	Lard.....
Lard.....	28.0	
	100.0	



the sole protein. The animal's life was terminated after 546 days of experiment. The quantities of feces from normally fed rats. See legend for Chart 1.

PERIOD 5.

per cent.		per cent.
18.0	Milk powder.....	60.0
28.2	Starch.....	12.0
30.8	Lard.....	28.0
5.0		
28.0		
		100.0
100.0		

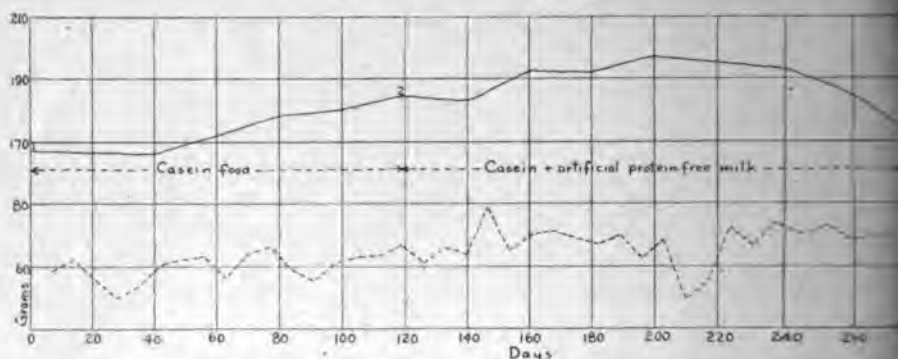


CHART 12, RAT 271 ♀, shows long-continued maintenance on a purely artificial diet containing casein as its sole protein. The animal died, after 277 days of experimental feeding, with diseased kidneys.

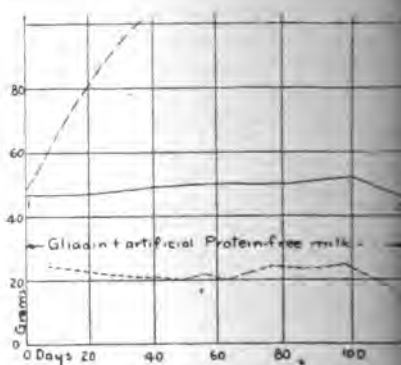
The diet during the different periods is shown below.

PERIOD 1.		PERIOD 2.	
	per cent.		per cent.
Casein (cow's milk).....	18.0	Casein (cow's milk).....	18.0
Starch.....	24.5	"Artificial" protein-free milk.....	28.0
Lactose.....	24.0	Starch.....	28.0
Agar.....	5.0	Lard.....	26.0
Salt mixture I.....	2.5		
Lard.....	26.0		
	100.0		

CHART 13, RAT 588 ♀, shows maintenance on a diet containing gliadin as the sole protein and an artificial imitation of the natural protein-free milk. After 114 days the artificial protein-free milk was replaced by the natural, but the decline in weight which had begun was not stopped by this change. The autopsy showed no adequate cause for death.

The diet in periods 1 and 2 was:

PERIOD 1.		PERIOD 2.	
	per cent.		per cent.
Gliadin.....	18.0	Gliadin.....	18.0
Artificial protein-free milk.....	30.0	Protein-free milk.....	28.0
Starch.....	22.0	Starch.....	26.0
Lard.....	30.0	Lard.....	28.0
	100.0		100.0



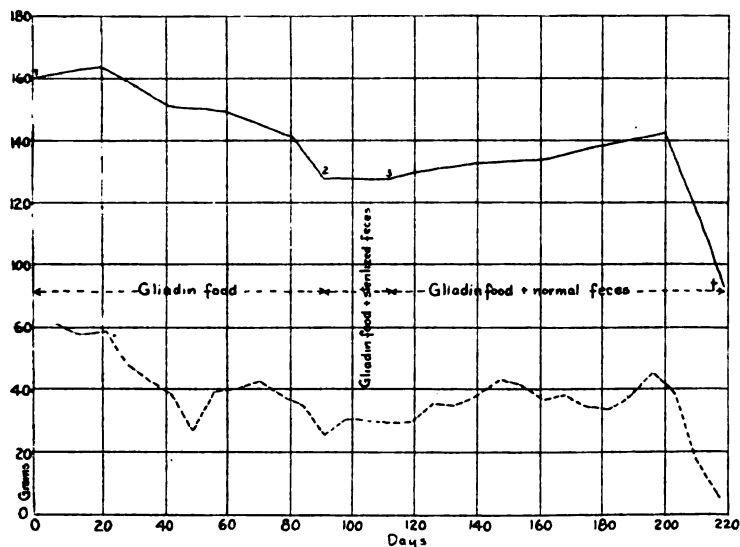


CHART 14, RAT 142 ♀, shows maintenance on a diet containing gliadin as its sole protein. During periods 2 and 3 the rat received small quantities of feces from normally fed rats. For a discussion of the effect of the sterilized and normal feces see Publication 156, p. 62, Carnegie Institution of Washington. The animal died suddenly after 219 days of experimental feeding, but unfortunately no autopsy was made.

The diet was as follows.

PERIODS 1, 2 AND 3.

	per cent.
Gliadin (wheat).....	18.0
Starch.....	29.5
Sucrose.....	17.0
Agar.....	5.0
Salt mixture I.....	2.5
Lard.....	28.0
	<hr/>
	100.0

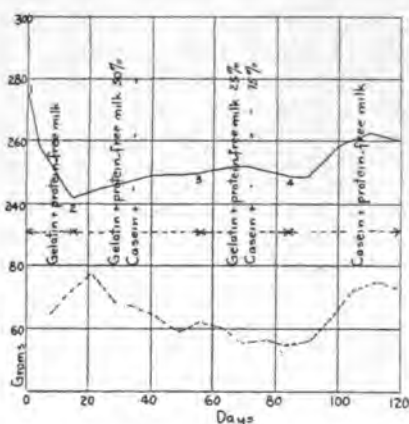


CHART 15, RAT 477 ♂, shows rapid decline on a diet containing gelatin as its sole protein, and subsequent maintenance and repair when the gelatin was either partially or entirely replaced by casein. After 158 days of experimental feeding the animal's life was terminated by diseased lungs.

The diet during the different periods is shown below.

PERIOD 1.

Gelatin food.

	per cent.
Gelatin (horn)....	18.0
Protein-free milk.	28.0
Starch.....	27.0
Lard.....	27.0

100.00

PERIOD 2.

per cent.

Gelatin food (as in period 1).....	50.0
Casein food (as in period 4).....	50.0
	100.0

PERIOD 3.

per cent.

Gelatin food (as in period 1).....	25.0
Casein food (as in period 4).....	75.0
	100.0

PERIOD 4.

Casein food.

	per cent.
Casein (cow's milk).....	18.0
Protein-free milk.	28.0
Starch.....	27.0
Lard.....	27.0

100.0

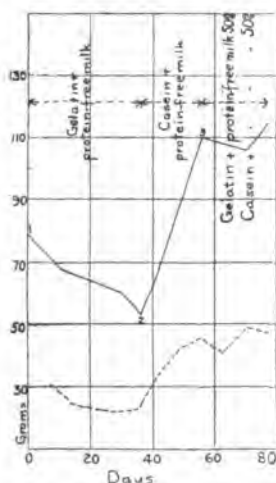


CHART 16, RAT 592 ♂, shows rapid decline on a diet containing gelatin as its sole protein, followed by normal growth when the gelatin is replaced by casein. The animal's life was terminated after 119 days of experimental feeding by calculi in the bladder.

The diet during the different periods is shown below.

PERIOD 1.

Gelatin food.

	per cent.
Gelatin (horn)....	18.0
Protein-free milk	28.0
Starch.....	27.0
Lard.....	27.0
	100.0

PERIOD 2

Casein food.

	per cent.
Casein (cow's milk).....	18.0
Protein-free milk.	28.0
Starch.....	27.0
Lard.....	27.0
	100.0

PERIOD 3.

per cent.

Gelatin food (as in period 1)....	50.0
Casein food (as in period 2).....	50.0

100.0

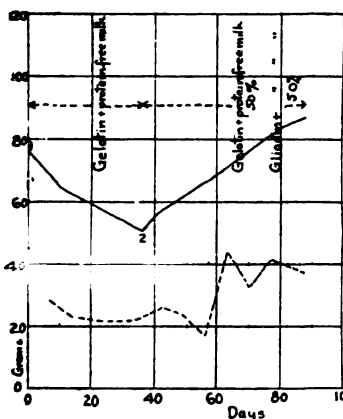


CHART 17, RAT 598 ♀, shows rapid decline on a diet containing gelatin as its sole protein, and recovery when one-half of the gelatin was replaced by gliadin, a protein incapable of inducing more than very slight growth when it forms the sole protein constituent of the dietary. The animal's life was terminated by diseased kidneys after 120 days of experimental feeding.

The diet during the different periods is shown below:

PERIOD 1.		PERIOD 2.		Gliadin food.	
	<i>per cent.</i>		<i>per cent.</i>		<i>gram.</i>
Gelatin (horn).....	18.0	Gelatin food (as in		Gliadin (wheat).....	18.0
Protein-free milk.....	28.0	period 1).....	50.0	Protein-free milk.....	28.0
Starch.....	27.0	Gliadin food.....	50.0	Starch.....	26.0
Lard.....	27.0			Lard.....	28.0
	<hr/>		<hr/>		
	100.0		100.0		100.0

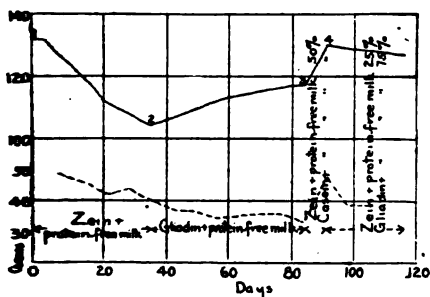


CHART 18, RAT 659 ♀, shows rapid decline on a diet containing zein as its sole protein, followed by recovery when the zein was entirely or partially replaced by gliadin or casein.

The diet during the different periods is shown below.

PERIOD 1.		PERIOD 2.		PERIOD 3.		PERIOD 4.	
	grams.		per cent.		per cent.		per cent.
Zein (maize).....	18.0	Glialin (wheat).....	18.0	Zein food (as in		Zein food (as in	
Protein-free milk.....	28.0	Protein-free milk.....	28.0	period 1).....	50.0	period 1).....	25.0
Starch.....	24.0	Starch.....	24.0	Casein food.....	50.0	Glialin food (as	
Lard.....	30.0	Lard.....	30.0			in period 2)....	75.0
					100.0		
	100.0		100.0				100.0
Water.....	15 cc.			Casein food,			
					grams.		
				Casein.....	18.0		
				Protein-free milk.....	28.0		
				Starch.....	29.0		
				Lard.....	25.0		
					100.0		

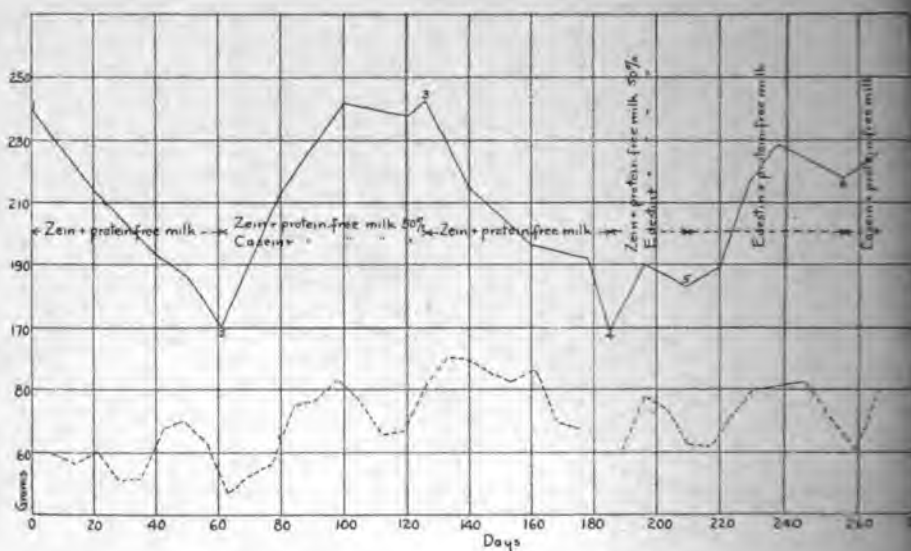


CHART 19, RAT 146 ♂, shows rapid decline on a diet containing zein as its sole protein followed by speedy recovery when the zein was partially or entirely replaced by casein or edestin. The experiment was terminated after 266 days of experimental feeding. The diet during the different periods is shown below.

PERIODS 1 AND 3.		PERIOD 2.		PERIOD 4.	
	grams.		per cent.		per cent.
Zein (maize).....	18.0	Zein food (as in period 1)	50.0	Zein food (as in period 1)	50.0
Protein-free milk.....	28.2	Casein food (as in period		Edestin food (as in period	
Starch.....	23.8	6).....	50.0	5).....	50.0
Agar.....	5.0				
Lard.....	25.0		100.0		100.0
	100.0				
Water.....	15 cc.				
PERIOD 5.		PERIOD 6.			
	per cent.		per cent.		
Edestin (hempseed).....	18.0	Casein (cow's milk).....	18.0		
Protein-free milk.....	28.2	Protein-free milk.....	28.2		
Starch.....	20.8	Starch.....	23.8		
Agar.....	5.0	Agar.....	5.0		
Lard.....	28.0	Lard.....	25.0		
	100.0		100.0		

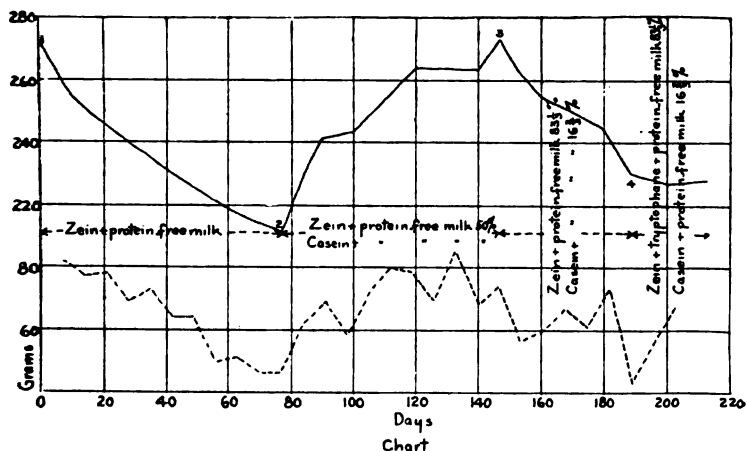


CHART 20, RAT 475 ♂, shows rapid decline on a diet containing zein as its only protein, followed by recovery when one-half of the zein was replaced by casein. Note that the decline in period 3 when only one-sixth of the zein was replaced by casein was immediately checked when 3 per cent of the zein was replaced by tryptophane.

This experiment is still in progress.

The diet during the different periods is shown below:

PERIOD 1.		PERIOD 2.		PERIOD 3.		PERIOD 4.	
	grams.		per cent.		per cent.		per cent.
Zein (maize).....	18.0	Zein food (as in		Zein food (as in		Zein food (as be-	
Protein-free milk	28.0	period 1).....	50.0	period 1).....	83.33	low).....	83.33
Starch.....	24.0	Casein food.....	50.0	Casein food (as		Casein food (as	
Lard.....	30.0			in period 2)....	16.67	in period 2)....	16.67
			100.0				
	100.0				100.00		100.00
Water.....	15 cc.						

Casein food.		Zein food.	
	grams.		grams.
Casein (cow's		Zein (maize).....	17.46
milk).....	18.0	Tryptophane.....	0.54
Protein-free milk	28.0	Protein-free milk	28.00
Starch.....	27.0	Starch.....	24.00
Lard.....	27.0	Lard.....	30.00
	100.0		100.00
		Water.....	15 cc.

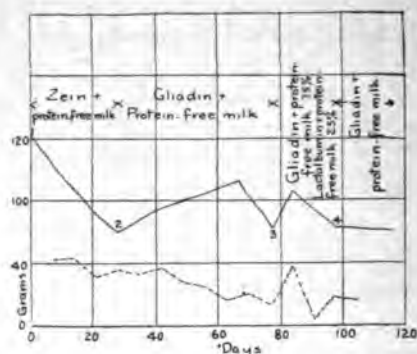


CHART 21, RAT 628 ♂, shows rapid decline on a diet containing zein as its sole protein, followed by maintenance when the zein was replaced by gliadin or lactalbumin.

The diet during the different periods is shown below.

PERIOD 1.		PERIODS 2 AND 4.		PERIOD 3.	
	grams.		per cent.		per cent.
Zein (maize).....	18.0	Gliadin (wheat)...	18.0	Gliadin food (as	
Protein-free milk	28.0	Protein-free milk	28.0	in period 2)....	75.0
Starch.....	24.0	Starch.....	26.0	Lactalbumin food	25.0
Lard.....	30.0	Lard.....	28.0		
	100.0		100.0		100.0
Water.....	15 cc.				

LACTALBUMIN FOOD.

	per cent.
Lactalbumin	
(cow's milk)....	18.0
Protein-free milk	28.0
Starch.....	29.0
Lard.....	25.0
	100.0

ON THE KYRINE FRACTION OBTAINED ON PARTIAL HYDROLYSIS OF PROTEINS.

FIRST COMMUNICATION.

BY P. A. LEVENE AND F. J. BIRCHARD.

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(Received for publication, October 3, 1912.)

Few facts of general character regarding the structure of the protein molecule have been added since the work of Fischer. The one very important contribution was that of Siegfried, based on his discovery of kyrines. The significance attributed to kyrines consisted in the hypothesis that they constituted fragments of the protein molecule, resembling natural protamines, and hence their discovery substantiated the theory of Kossel that protamines represent the nucleus to which other amino-acids join on in order that a complex molecule of protein may be formed. Whether or not Siegfried has proven Kossel's hypothesis is a problem still awaiting solution, but this investigator undoubtedly has indicated a process by which can be accomplished a partial hydrolysis of protein, leading to the formation of comparatively simple peptides. *A priori*, it seemed logical to expect that if more than one-basic peptides were formed in the process of hydrolysis they would all appear in the general fraction named by Siegfried "kyrine sulphate." Hence it seemed desirable to investigate more closely the composition of the kyrine fraction obtained on partial hydrolysis of protein with the view of a possible discovery in it of more than one peptide.

The expectation was realized inasmuch as the crude kyrine fraction obtained from gelatin was fractionated into two peptides. This conclusion has forced itself on us first as a result of the comparison of the ratio $\frac{\text{Amino N}}{\text{Total N}}$ before and after hydrolysis of the kyrine mixture. The arguments can be made more obvious by discussing the details of the analytical data.

Following the directions of Siegfried, a substance was obtained from gelatin, resembling in every way kyrine sulphate, which was further purified by means of silver sulphate and barium hydrate. The substance obtained after this purification had the following properties: Its elementary composition resembled that of Siegfried's kyrine; its phosphotungstic compound crystallized in the same form as described by Siegfried for the same derivative of kyrine; on hydrolysis it gave arginine, lysine, glutamic acid, glycocoll and proline. The presence of the latter was demonstrated by the indirect method. These amino-acids were combined in peptide linking to some extent. The reason for this assumption is the following: If the substance were a mixture of amino-acids the ratio of the amino nitrogen to the total nitrogen should have been 5:9. Since in arginine three nitrogen atoms do not function as primary amino nitrogen, and since the nitrogen of proline must be placed in the same class, in a mixture of the five named amino-acids containing nine nitrogen atoms, five possess the properties of primary amines in their behavior to nitrous acid.

The ratio of amino nitrogen to total nitrogen in our substance was only from 32 to 33 per cent, indicating that some of the primary amino nitrogen atoms had ceased to function as such, presumably through the formation of peptide linkings.

The next task was to ascertain whether or not the five amino-acids were combined in one or in more than one peptide. If the five acids were linked into one peptide this should contain only two nitrogen atoms in the form of primary amino nitrogen, namely, that of the end-acid and the one in the ω -position in the lysine. Thus a pentapeptide composed of the five named amino-acids requires a ratio of amino nitrogen to total nitrogen of 2:9 or of 22 per cent. The substance obtained by us contained 32 per cent of amino nitrogen. Hence it was composed of more than one peptide.

These data, and the data obtained on the nitrogen partition after hydrolysis, led us to the belief that the substance consisted of two basic peptides: one containing lysine and three mono-amino-acids, and the other arginine and one amino-acid. This assumption was corroborated by the fact that it was possible to separate the mixture into two fractions by means of silver sul-

phate and barium hydrate. One of these, arginine glutaminic acid peptide, was about 95 per cent pure. The degree of purity was demonstrated by the ultimate analysis of its sulphate, by the ratio of amino nitrogen before and after hydrolysis, by the quantitative estimation of arginine contained in it and by the fact that both arginine and glutaminic acid were obtained from it on hydrolysis. The second peptide has not yet been obtained in the same degree of purity. The nitrogen partition of the pure peptide requires a ratio of 40 per cent of amino nitrogen; instead of which, the substance contained only about 37 per cent. It is hoped that this peptide will also be obtained in a sufficient degree of purity before very long. Work on the separation of the basic peptides from other proteins is at present in progress.

The substance discussed here, with the $\frac{\text{Amino N}}{\text{Total N}}$ ratio of 32 per cent was not exactly the kyryne sulphate of Siegfried, but was chosen for the reason that it offered a more convenient material for presenting our arguments. The kyryne prepared according to Siegfried by repeating the precipitation with alcohol five times had the ratio $\frac{\text{Amino N}}{\text{Total N}} = 23.6$ per cent. By means of one treatment with silver sulphate and barium hydrate it was separated into two fractions, one of which consisted principally of the dipeptide, the other being a mixture having the ratio $\frac{\text{Amino N}}{\text{Total N}} = 32$ per cent. Hence it is evident that the substance obtained by us according to the directions of Siegfried contained a higher proportion of the dipeptide than the mixture discussed in this communication.

However, it should be born in mind that only slight changes in the conditions of hydrolysis may bring about considerable variations in the composition of the products obtained through it, and hence it is possible that the substance analyzed by Siegfried had a composition different from that of the substance obtained by us.

EXPERIMENTAL PART.

The original material was prepared according to the method of Siegfried for the preparation of gluto-kyrine¹ and need not be discussed here. It was analyzed as sulphate after drying in vacuum to constant weight at the temperature of boiling chloroform for one day and at the temperature of boiling alcohol for seven days, and had the following composition.

Ash, 1.8 per cent.

Substance, 0.2250 gram; CO₂, 0.2948 gram; H₂O, 0.1248 gram.

Substance: 0.1538 gram required 17.65 cc. of $\frac{N}{10}$ acid.

Substance: 0.2618 gram gave 0.2032 gram BaSO₄.

	Substance here described:	Kyrine sulphate of Siegfried:
C.	35.70 per cent.	31.86 per cent.
H.	6.22 per cent.	5.61 per cent.
N.	16.06 per cent.	15.98 per cent.
S.	10.65 per cent.	10.13 per cent.

Amino nitrogen ratio in the original kyrine sulphate.

The ratio of amino nitrogen to total nitrogen was determined according to the method of Van Slyke. One gram of substance was dissolved in 50 cc. of water and the total nitrogen determined according to Kjeldahl and the amino nitrogen according to Van Slyke.

Total N: 10 cc. solution neutralized 21.8 cc. $\frac{N}{10}$ acid. N = 30.52 mgm.

Amino N: 10 cc. solution gave 12.7 cc. gas at 19°, 754 mm. N = 7.20 mgm.

Amino N
Total N 23.61 per cent.

The substance was ammonia-free. 0.5 gram substance distilled with Ca(OH)₂ and methyl alcohol under diminished pressure neutralized 0.0 cc. $\frac{N}{10}$ acid.

Purification of the kyrine sulphate.

The kyrine prepared according to Siegfried's directions was further purified by treatment with silver sulphate and barium

¹ Bericht der math.-physik. Klasse der königl. sächsischen Gesellschaft der Wissenschaften zu Leipzig, March 2, 1903.

hydrate as in Kossel's method for the separation of arginine from the hydrolytic mixture. The kyrrine sulphate was dissolved in a few cubic centimeters of water and a hot solution of silver sulphate added, care being taken to constantly cool the resulting liquid by surrounding it with ice. The addition of silver sulphate was continued until a drop of the mixture added to a solution of barium hydrate on a watch glass produced a brown coloration. After cooling, finely powdered barium hydrate was slowly added to complete saturation, the liquid being constantly stirred mechanically. The precipitate was filtered off with the aid of suction, the whole being kept in a refrigerator at approximately 0°. The precipitate and the filtrate were then slightly acidified with sulphuric acid and decomposed with hydrogen sulphide. The resulting solutions were finally freed from sulphuric acid, concentrated to a small volume under diminished pressure, the sulphates formed by the addition of sulphuric acid and each precipitated in a large excess of absolute alcohol. After standing for some time in the refrigerator at 0° the peptides were filtered off with the aid of suction, washed with absolute alcohol and ether and dried in a desiccator over sulphuric acid.

Nitrogen partition in the "purified kyrine" before hydrolysis.

0.75 gram substance was hydrolyzed with 20 per cent hydrochloric acid for twenty hours. The excess of hydrochloric acid was then distilled off under diminished pressure, the residue taken up in water, exactly neutralized with sodium hydrate and finally brought to a volume of 50 cc. In a duplicate experiment 1.4 grams of substance were treated in the same manner.

- (1) Total N: 10 cc. solution required 15.8 cc. $\frac{N}{10}$ acid.
 N = 22.12 mgm.
 Amino N: 10 cc. solution gave 12.8 cc. gas, 19°, 760 mm.
 N = 7.28 mgm.

<u>Amino N</u>	
Total N	32.9 per cent.

- (2) Total N: 10 cc. solution required 38.7 cc. $\frac{N}{10}$ acid.
N = 54.21 mgm.
Amino N: 10 cc. solution gave 31.5 cc. gas, 25°, 758 mm.
N = 17.46 mgm.

<u>Amino N</u>	
Total N	32.2 per cent.

The ratio of amino nitrogen to total nitrogen after hydrolysis.

0.5 gram of the substance was hydrolyzed with 30 cc. of a 20 per cent solution of hydrochloric acid for twenty hours over flame with return condenser. The excess of hydrochloric acid was removed by distillation, the residue taken up in water, neutralized with sodium hydrate and brought to a volume of 50 cc.

Total N: 10 cc. solution required 12.89 cc. $\frac{N}{10}$ acid.

N = 18.04 mgm.

Amino N: 10 cc. solution gave 20.4 cc. gas, 19°, 760 mm.

N = 11.69 mgm.

Amino N	
Total N	64.7 per cent.

Nitrogen partition in the phosphotungstic acid precipitate and filtrate after hydrolysis.

One gram of substance was hydrolyzed with a 20 per cent solution of hydrochloric acid for twenty hours over flame with return condenser. After removing the hydrochloric acid by repeated concentration under diminished pressure the residue was taken up with 10 per cent sulphuric acid and a 10 per cent solution of phosphotungstic acid added. The solution and precipitate so formed were then heated on the water bath until the precipitate was nearly all dissolved and then allowed to stand until the next day. The nitrogen in the precipitate and filtrate was determined according to Kjeldahl.

The precipitate required 44.60 cc. of $\frac{N}{10}$ acid and the filtrate 25.92 cc. of $\frac{N}{10}$ acid. Hence the ratio of nitrogen in the phosphotungstic precipitate to the total nitrogen = $\frac{44.60}{70.52} = 63.25$ per cent.

Amino nitrogen ratio in the phosphotungstic acid precipitate and filtrate.

In a second portion the ratio of amino nitrogen to total nitrogen in the phosphotungstic acid precipitate and filtrate after acid hydrolysis was determined. The precipitate was brought into solution with the aid of dilute alkali and brought to a volume of 30 cc.

Total N: 10 cc. solution gave 9.19 cc. $\frac{N}{10}$ acid. N = 38.59 mgm.
in toto.

Amino N: 10 cc. solution gave 12 cc. gas, 20°, 760 mm. N = 20.52
mgm. in toto.

Amino N	
Total N	53.1 per cent.

The filtrate was concentrated to a volume of 50 cc.

Total N: 35 cc. solution required 15 cc. $\frac{N}{10}$ acid. N = 30.0 mgm.
in toto.

Amino N: 10 cc. solution gave 7.9 cc. gas, 19°, 760 mm. N = 22.53
mgm. in toto.

Amino N	
Total N	75.1 per cent.

Hydrolysis of the kyrine fraction aiming to isolate the individual components.

About 45 grams of the sulphate were hydrolyzed with a 20 per cent solution of hydrochloric acid for twenty-four hours. The solution was then treated with phosphotungstic acid in the manner before described, the precipitate was washed chlorine-free with 5 per cent sulphuric acid, and the phosphotungstic and sulphuric acids were removed quantitatively with barium hydrate. The resulting liquid was concentrated to a small volume and to a portion an alcoholic solution of picronic acid was added and allowed to stand over night. The precipitate so obtained was recrystallized out of water and analyzed.

Substance, 0.1600 gram; CO₂, 0.2541; H₂O, 0.0706.

	Calculated for C ₆ H ₁₄ N ₂ O ₂ · C ₁₀ H ₈ N ₄ O ₈	Found:
C.....	43.82	43.23
H.....	5.05	4.90

Lysine was isolated from the phosphotungstic acid precipitate according to Kossel's method and obtained as the picrate. It was identified by determining the amino nitrogen.

Substance, 0.0930 gram; gas, 12.8 cc., 20°, 764 mm.

	Calculated for C ₆ H ₁₃ N ₂ O ₂ · C ₆ H ₇ (NO ₂) ₃ OH:	Found:
Amino N.....	7.48	7.86

Glutaminic acid was separated from the phosphotungstic acid filtrate as hydrochloride after removal of the sulphuric and phosphotungstic acids quantitatively by barium hydrate.

0.1500 gram substance gave 0.1153 gram silver chloride.

	Calculated for $C_4H_9NO_4 \cdot HCl$	Found:
Cl.....	19.31	19.01

The excess of hydrochloric acid was removed by concentration under diminished pressure and the remainder with silver sulphate. The silver was removed with hydrogen sulphide and the sulphuric acid with barium hydrate and the volume brought to 200 cc.

Total N: 5 cc. required 17.85 cc. $\frac{N}{10}$ acid. N = 24.94 mgm.

Amino N: 5 cc. gave 32.2 cc. gas, 20°, 760 mm. N = 18.30 mgm.

The ratio of amino nitrogen to total nitrogen in the phosphotungstic acid filtrate = 73.3 per cent or 26.7 per cent of the nitrogen is non-amino nitrogen. This indicates the presence of proline or oxyproline.

The copper salts were formed from the remainder of the filtrate and extracted with hot alcohol. On standing a large portion crystallized out. The portion soluble in hot and cold alcohol appeared on analysis to be largely the copper salt of proline or oxyproline. It was dissolved in 20 cc. of water.

Total N: 10 cc. required 20.43 cc. $\frac{N}{10}$ acid. N = 28.60 mgm.

Amino N: 10 cc. gave 17 cc. gas, 18°, 764 mm. N = 9.81 mgm.

$\frac{\text{Amino N}}{\text{Total N}} = 34.3$ or 65.7 per cent of the nitrogen of the phosphotungstic acid filtrate is non-amino.

The nitrogen content of the crystals which separated from the hot alcohol did not correspond to any of the ordinary amino acids.

0.0964 gram substance required 7.3 cc. $\frac{N}{10}$ acid. N = 10.22 mgm.

Total N.....10.60 per cent.

0.0991 gram substance gave 18.4 cc. gas, 20°, 764 mm.

N = 10.53 mgm.

Amino N.....10.63 per cent.

The analysis of the copper salt of the portion insoluble in hot and cold alcohol indicated a mixture, probably of alanine and glycocoll.

0.2154 gram substance gave 0.2146 gram CO_2 ; 0.0910 gram H_2O .

	Calculated for $(\text{C}_2\text{H}_4\text{NO}_2)_2\text{Cu}$:	Calculated for $(\text{C}_2\text{H}_5\text{NO}_2)_2\text{Cu}$:	Found:
C.....	25.00	30.00	27.28
H.....	4.16	5.00	4.60

The results of the hydrolysis show that arginine, lysine and glutaminic acid are certainly present and probably also proline. Glycocoll may also be present and perhaps alanine.

Preparation of the dipeptide.

The precipitate obtained on treatment of the solution of the original kyrine with silver sulphate and barium hydrate contained the dipeptide. It was freed from silver and barium in the manner described earlier in the paper and transformed into the sulphate in the same manner as the original kyrine. The dipeptide nature of the substance was based on the $\frac{\text{Amino N}}{\text{Total N}}$ ratio before and after

hydrolysis, on the fact that on hydrolysis it yielded arginine and glutaminic acid and on its elementary composition. A dipeptide composed of arginine and glutaminic acid should contain only one free primary amino group out of five nitrogen atoms in the molecule, or the $\frac{\text{Amino N}}{\text{Total N}}$ is calculated at 20 per cent. The values found on analysis of the substance only slightly exceeded that value.

The ratio of the nitrogen in the form of amino-acids to the basic nitrogen is calculated also at 20 per cent, and the analyzed body showed the value of the nitrogen of amino-acids to represent 21.1 per cent of the total nitrogen.

Furthermore, on hydrolysis of the dipeptide, free arginine and free glutaminic acid are formed, hence the mixture of the two contains twice the number of amino groups as compared with the original number. The value found on hydrolysis of the analyzed body only slightly exceeded the theoretical value.

Finally the arginine determination made directly on the peptide gave results deviating only slightly from the value theoretically calculated.

Elementary analysis of the dipeptide.

The dipeptide was analyzed as sulphate. It was dried under diminished pressure at room temperature over sulphuric acid for several days and then at the temperature of boiling chloroform for two days and at that of boiling carbon tetrachloride for five days.

Substance, 0.2407 gram; CO_2 , 0.2943; H_2O , 0.1242.

Substance, 0.1808 gram; CO_2 , 0.2160; H_2O , 0.0916.

0.3209 gram substance required 40.1 cc. $\frac{\text{N}}{10}$ acid = 56.14 mgm. N.

0.4033 gram substance gave 0.2712 gram BaSO_4 .

0.1952 gram substance gave 0.1276 gram BaSO_4 .

	Calculated for ($\text{C}_{10}\text{H}_{15}\text{N}_3\text{O}_4$) $\cdot\text{H}_2\text{SO}_4$:	Found:
C.....	32.90	32.97
H.....	5.73	5.72
N.....	17.40	17.48
S.....	8.20	9.11

Hydrolysis of the dipeptide indicating the nitrogen partition before and after hydrolysis.

Two solutions of the sulphate were prepared, the first containing approximately 0.5 gram of the sulphate in 25 cc. and the second, 1.0 gram in 25 cc. These solutions were used for the determination of the amino nitrogen before hydrolysis. A third solution containing approximately 2.5 grams of the sulphate was hydrolyzed with hydrochloric acid in the usual manner and the nitrogen partition in the phosphotungstic acid precipitate and filtrate determined in the manner described for the "purified kyrine" fraction. The ratio of amino nitrogen to total nitrogen was also determined in the same manner.

Before hydrolysis.

(a) Total N: 10 cc. solution required 17 cc. $\frac{\text{N}}{10}$ acid. N = 23.80 mgm.

Amino N: 10 cc. solution gave 8.7 cc. gas, 20° , 760 mm. N = 4.96 mgm.

Amino N

..... 20.8 per cent.

Total N

(b) Total N: 5 cc. solution required 21.4 cc. $\frac{\text{N}}{10}$ acid. N = 29.96 mgm.

Amino N: 10 cc. solution gave 22.5 cc. gas, 30° , 758 mm. N = 12.23 mgm.

Amino N

..... 20.4 per cent.

Total N

After hydrolysis.

(1) *Nitrogen partition in phosphotungstic acid precipitate and filtrate.*

Total volume of the solution, 50 cc. 5 cc. solution required 21.78 cc. $\frac{N}{10}$ acid. Total N = 304.92 mgm.

Total N in filtrate from phosphotungstic acid precipitate: Total volume of solution, 100 cc. 25 cc. solution required 11.55 cc. $\frac{N}{10}$ acid. N = 16.17 mgm. Total N = 64.68 mgm.

The ratio of the nitrogen in the phosphotungstic acid filtrate to the total nitrogen = 21.1 or the ratio of the nitrogen in the phosphotungstic acid precipitate to the total nitrogen = 78.9 per cent.

Allowing for the correction of the solubility of arginine phosphotungstate, one finds the nitrogen value of the phosphotungstic precipitate to constitute 79.7 per cent of the total nitrogen.

(2) *The ratio of amino nitrogen to total nitrogen.* This determination was made on a second portion hydrolyzed as previously described.

Total N: 10 cc. solution required 21.90 cc. $\frac{N}{10}$ acid. N = 30.66 mgm.

Amino N: 10 cc. solution gave 24 cc. gas, 20°, 760 mm. N = 13.68 mgm.

Amino N	
Total N	44.4 per cent.

Hydrolysis of the dipeptide.

100 cc. of an aqueous solution of the dipeptide containing 1.22 grams of nitrogen were concentrated to 20 cc. and 2 grams of sulphuric acid added. This was hydrolyzed by heating for five hours in a sealed tube. Water was added so that the solution finally contained 5 per cent sulphuric acid and then was precipitated with phosphotungstic acid. After standing for two days in the refrigerator, the precipitate was filtered off in the cold and washed with 5 per cent sulphuric acid. The phosphotungstic acid was removed from the filtrate by shaking out with ether, the ether distilled off, and the sulphuric acid removed quantitatively with barium hydrate. The resulting solution was concentrated under diminished pressure to a small volume and alcohol added, when a portion crystallized out. This crystallized portion was dissolved in water and converted into the picrolonate and analyzed.

Substance, 0.0998 gram; CO₂, 0.1604 gram; H₂O, 0.0338 gram.

	Calculated for C ₆ H ₇ NO ₄ · C ₁₀ H ₈ N ₂ O ₃ :	Found:
C.....	43.75	4.38
H.....	4.16	3.80

The mother liquors were also converted into the picrolonate. These analyzed for a mixture of an inorganic picrolonate and impure glutaminic acid.

Ash-free substance, 0.1038 gram; CO₂, 0.1702 gram; H₂O, 0.0274 gram; Ash, 0.0022 gram.

C.....	44.70 per cent.
H.....	2.95 per cent.
Ash.....	2.20 per cent.

The phosphotungstic acid precipitate was suspended in water, decomposed with barium hydrate and the acid removed quantitatively. The resulting solution was concentrated under diminished pressure and picrolonic acid added. The resulting crystals were filtered off, washed, dried and analyzed.

Substance, 0.1196 gram; CO₂, 0.1804 gram; H₂O, 0.0420 gram.

	Calculated for C ₆ H ₁₁ N ₄ O ₅ (C ₁₀ H ₈ N ₂ O ₃) ₂ :	Found:
C.....	44.40	45.05
H.....	4.27	4.30

Arginine content of the dipeptide.

The arginine was determined directly on the peptide without preceding hydrolysis. 10 cc. of an aqueous solution of the substance were boiled in a Kjeldahl flask with 20 cc. water and 15 grams of potassium hydrate in the manner described by Van Slyke² for arginine determination. At the end of ten hours water was added to the flask and the distillation continued for another hour. Of the original aqueous solution of the dipeptide 10 cc. were used for the arginine determination and 5 cc. for the total nitrogen.

Total N: 5 cc. solution required 35 cc. $\frac{N}{10}$ acid. N = 49 mgm.

Arginine N: 10 cc. solution boiled with 20 cc. water and 15 grams

KOH neutralized 26.75 cc. $\frac{N}{10}$ acid = 37.45 mgm.

N. Total arginine N = 74.90 mgm.

	Calculated:	Found:
Total Arginine N.....	80.0	76.5 per cent.

² This *Journal*, x, p. 26, 1911.

Amino nitrogen content in the mother liquor from the dipeptide.

On the basis of the assumption made in the present communication, the filtrate from the silver and barium precipitate should contain a peptide composed of all the other amino-acids recognized among the cleavage products of the original kyrine, except arginine and glutaminic acid. The polypeptide formed in this manner should contain two amino groups and three non-amino nitrogen atoms in the molecule or $\frac{\text{Amino N}}{\text{Total N}}$ ratio = 40 per cent. We have not yet succeeded in obtaining a solution of the pure tetrapeptide; however, after five repeated treatments with silver sulphate and barium hydrate the $\frac{\text{Amino N}}{\text{Total N}}$ ratio of the solution rose from 23 per cent to 36 per cent, thus indicating that with more patience and with more material one might obtain the pure tetrapeptide.

Total N: 5 cc. solution required 16.5 cc. $\frac{N}{10}$ acid. N = 23.10 mgm.

Amino N: 10 cc. solution gave 29.0 cc. gas, 21°, 772 mm.

N = 8.34 mgm.

$\frac{\text{Amino N}}{\text{Total N}}$ 36.1 per cent.







A CONTRIBUTION TO OUR KNOWLEDGE OF THE GAS METABOLISM OF BACTERIA.

FIRST PAPER.

THE GASEOUS PRODUCTS OF FERMENTATIONS OF DEXTROSE BY *B. COLI*, BY *B. TYPHOSUS* AND BY *BACT. WELCHII*.

By FREDERICK G. KEYES AND LOUIS J. GILLESPIE.

(From the Biological Laboratory of Brown University.)

(Received for publication, October 9, 1912.)

Earlier investigations.

B. coli. The literature on the gases produced by *B. coli* on various culture media has already been reviewed by one of us.¹ It was found that very little work had been attempted on this subject by investigators familiar with the properties of gases.

Harden² found that, when *B. coli* was grown anaerobically on a non-albuminous dextrose medium based on asparagine, the asparagine was reduced to ammonium succinate with a consequent lessened evolution of hydrogen. He therefore made use of media made of beef broth and of Witte's peptone and concluded, from a study of the solid, liquid and gaseous products, that on these media dextrose was decomposed with the formation (if not evolution) of equal volumes of hydrogen and of carbon dioxide. His gas analyses showed for the most part more hydrogen than carbon dioxide.

One of us³ determined for several strains of *B. coli* the quantities and the composition of gas obtained in various incubation periods

¹ Keyes: *Journ. of Med. Res.*, xxi (N. S. xvi), p. 69, 1909. In this communication the composition of the culture medium was unfortunately given incorrectly. The medium always contained, in addition to the constituents noted, 1 per cent of Merck's "Highest Purity" dextrose.

² Harden: *Trans. Jenner. Inst.*, 1899, ii, p. 126; *Journ. Chem. Soc. (Transactions)*, lxxix, p. 610, 1901.

³ Keyes: *Journ. of Med. Res.*, xxi (N. S. xvi), p. 69, 1909.

by an exact method from vacuum fermentations of dextrose. A non-albuminous asparagine medium was used for the most part, but a dextrose meat bouillon was also tested, and it was found that a larger percentage of hydrogen was indeed evolved from the albuminous medium but that even on this medium carbon dioxide was produced in considerably greater volume than hydrogen.⁴

B. typhosus. Hesse⁵ studied the gas metabolism of this organism by growing it in the presence of air and drawing off samples of gas from day to day for analysis. He stated that carbon dioxide is evolved and that oxygen is absorbed but he did not calculate his analytical results. We calculate from his protocols that in one instance as much as 18 cc. of carbon dioxide was evolved aërobically from 33 cc. of "glycerin agar-agar."

Harden⁶ detected no production of gas from anaërobic fermentations of dextrose by *B. typhosus* and Pakes and Jollyman⁷ detected no formation of gas from sodium formate—the supposed intermediate product between dextrose and carbon dioxide and hydrogen.

Bact. welchii. Dunham⁸ analyzed the gas evolved by this organism from 1 per cent dextrose bouillon in Smith fermentation tubes. The ratio $\text{CO}_2:\text{H}_2$ was 0.43, but his results are not comparable with ours because he disregarded the loss of carbon dioxide due to solution in the medium and to diffusion from the open end of the fermentation tube.

Technique.

Organisms. Cultures were kept in a vigorous state of growth by means of frequent transfers and isolations by the plate method. The medium used for *B. typhosus* and *Bact. welchii* was the ordinary meat extract peptone agar; in the case of *B. coli* it consisted

⁴ We shall give below an analysis from a fermentation on a Witte's peptone medium, which gave practically the same figures as the meat bouillon. We have always found more CO_2 than H_2 , and attribute Harden's contrary result, as also his negative result with *B. typhosus*, to insufficient removal of dissolved carbon dioxide from the culture fluid.

⁵ Hesse: *Zeitschr. f. Hyg.*, xv, p. 17, 1893.

⁶ Harden: *Journ. Chem. Soc. (Transactions)*, lxxix, p. 610, 1901.

⁷ Pakes and Jollyman; *Journ. Chem. Soc. (Transactions)*, lxxix, p. 459, 1901.

⁸ Dunham: *Johns Hopkins Hospital Bull.*, viii, p. 68, 1897.

of 1 per cent dextrose, 0.2 per cent disodium phosphate and 1.5 per cent agar, with either 1 per cent asparagine for experiments on asparagine media or 1 per cent ammonium lactate for experiments on lactate media, and was made neutral to litmus with sodium hydrate. Test cultivations of *B. coli* or of *B. typhosus* were started from a loopful of culture taken from the surface of a twenty-four-hour growth on slant agar. *Bact. welchii* fermentations were started from stab cultures. Subcultures from the test cultivations were never used for further tests. The organisms answered the usual identification tests.

Culture fluids. The culture fluids were sterilized in the absence of air in fermentation bulbs (to be described) in streaming steam by the intermittent method. The reaction after boiling was slightly acid to litmus, except in certain experiments (noted in the protocols) in which the reaction was made just neutral to phenolphthalein by the addition of normal sodium hydrate. When alkali was added, the amount of carbon dioxide thus introduced was calculated from a determination of the actual volume of carbon dioxide liberated from a sample of the sodium hydrate solution upon acidification *in vacuo* with sulphuric acid. Since the medium was subsequently exposed only momentarily to the atmosphere, the correction applied (a small one) was exact.

Oxygen. Oxygen was prepared by heating potassium permanganate *in vacuo* and was purified by passing over phosphorus pentoxide and over sodium hydrate (not that purified by alcohol). It was measured dry, the pressure being read on a barometer column. Generator, burette and barometer were permanently incorporated in the pump system (fig. 2) by fused glass joints. The generator was exhausted by the mercury pump and then rinsed out twice with small quantities of oxygen before the gas was generated for use in the work.

Control of gaseous environment and collection and analysis of the gases. Fermentations were conducted either *in vacuo*⁹ or in the presence of gases admitted in known quantities after a vacuum had first been obtained.

The fermentation bulbs (fig. 1) fitted with stopcocks of a special form described by one of us,¹⁰ were about half or three-quarters filled with cul-

⁹ That is, in an atmosphere of water vapor only.

¹⁰ Keyes: *Science* (N.S.), xxviii, p. 17, 1908.

ture medium, and were sterilized. After sterilization, the stopcocks of the bulbs were wiped dry with sterile rolls of filter paper and re-greased with sterile lubricant. The lubricant was the non-volatile non-antiseptic mixture described by one of us.¹¹ The medium was then inoculated.

The bulbs were then freed from gases. It was found that this could be done to any desired degree of completion by the use of a good Sprengel water pump, which was capable of maintaining the pressure at from 9 to 15 mm. (The use of a mercury pump gave no better results and was moreover unnecessarily troublesome.) This method of exhaustion depends upon rinsing out the gas with water vapor. In extracting dissolved gases from liquids the driving force becomes less as the process becomes more nearly complete and the process comes practically to an end while detectable amounts of gases are still dissolved, unless by vigorous agitation the liquid is kept well mixed and many new boundary surfaces thus continually formed. We found that, without agitation, pumping for an hour after most of the air had been removed was not so efficacious as five or ten minutes' vigorous shaking interrupted by a few exposures to the action of the pump. This experience led us to think that the degree of exhaustion is controlled, in cases where the space above the liquid is kept as free from gases as in these experiments, by the thoroughness of agitation. The bulbs were therefore subjected alternately to the action of the pump, by opening the stopcock of the bulb for an instant, and to a vigorous shaking. This was repeated until a metallic sound was emitted upon striking the bulb hard with the finger ends¹² and was then further continued until this effect (which cannot be produced until the vacuum is rather good) was produced only with great difficulty. As the liquid was shaken, falling drops of liquid clicked metallically against the sides of the bulb. We believe that we were able to treat the bulbs with practical uniformity and that before we finished the process the gas that remained in any bulb was negligible in quantity so far as our analyses were concerned.¹³

If gas was to be admitted, the bulb was now connected to the pump system by a short piece of heavy walled rubber tubing, which was well painted with hot adherent grease. Both ends of the tube were bound tightly on with copper wire. The tubing connecting the bulb with the gas burette was evacuated by the use of the mercury pump, and the gas, having been measured, was pushed over into the bulb. By careful manipulation practically all the gas could be passed over into the bulb without admitting any mercury.

¹¹ Keyes: *Journ. Amer. Chem. Soc.*, xxxi, p. 1271, 1909.

¹² Special experiments have shown us that this sound is occasioned by the tearing apart of the liquid with the liberation of a bubble or two of gas and the consequent collapse of the newly formed walls of liquid. Thus the range of concentrations of dissolved gases at which this effect is possible, should be, as it in fact is, limited in both directions.

¹³ Except possibly in the case of *B. typhosus*, where the total gas evolution was very small.

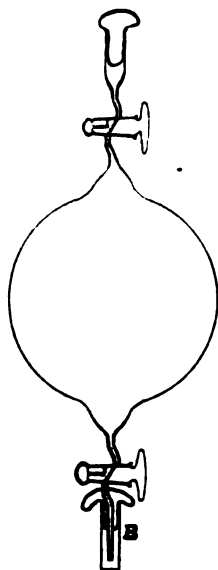


FIG. 1. FERMENTATION BULB
FITTED WITH VACUUM
STOPCOCKS.

B, bottle, secured by cotton plug, serving to keep sterile the capillary lead. Capacity of bulb, about 300 cc.

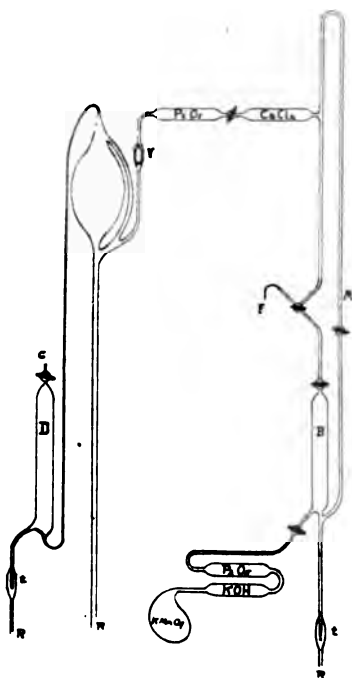


FIG. 2. THE PUMP SYSTEM.

B, oxygen measuring burette; *D*, delivery tube—it connects with gas analysis apparatus at *C*; *M*, manometer column, provided with meter stick; *t, t*, air traps; *V*, valve tight to ascending mercury. Fermentation bulb connects at *F*. Mercury reservoirs connect by heavy rubber tubing at *R, R, R*.

After incubation at about 37°C., the gases in the bulb were recovered by the mercury pump and analyzed. In every instance, the whole obtainable quantity was pumped out and the whole quantity was used for the analysis or else a measured portion after the gases were well mixed. The gas absorptions were conducted according to the method of Hempel. The burettes were filled with mercury. Carbon dioxide was absorbed by strong caustic soda solution (after the total volume had been measured dry); oxygen, by phosphorus or when necessary by alkaline pyrogallol; and hydrogen

was determined by exploding with a quantity of air sufficient to avoid the burning of nitrogen. Other gases were absent. Sometimes the Hempel pipettes were used for the reagents; but when small amounts of gas were to be analyzed, they were generally measured under diminished pressure, and treated with absorbing reagents in tubes inverted over mercury, as in the method given by Travers.¹⁴ Sometimes the diminution of pressure was read on an open manometer; and sometimes the actual pressure was read on a barometer (the space of which was saturated with water vapor) sealed to the burette. The work was done in a cellar where the temperature was sufficiently constant. All gas volumes have been calculated for 0°C., 760 mm. and dryness. The medium was always strongly acid after incubation so that no carbon dioxide was retained chemically.

Experiments with B. Coli.

Experiment 1. *B. coli* was grown in *vacuo* for 48.2 hours on 250 cc. of a medium containing 1.00 per cent each of Witte's peptone and Merk's "Highest Purity" dextrose in distilled water, prepared hot and filtered.

Total gas.....	137.0 cc.
CO ₂	56.1 per cent.
H ₂	43.0 per cent.
CO ₂ per gram of dextrose.....	30.7 cc.
Ratio, CO ₂ :H ₂	1.31

The ratio 1.28 was found by one of us¹⁵ for beef infusion broth containing 1 per cent each of dextrose and Witte's peptone.

It seemed better to work with a medium containing no substances of unknown composition. One such, described by Dolt,¹⁶ has the advantage that no amino group is present so that retention of hydrogen by the type of reaction found by Harden for asparagine is excluded. It consisted of 1.00 per cent ammonium lactate, 1.00 per cent dextrose and 0.200 per cent disodium phosphate in distilled water, prepared without heating. In table I are given some experiments upon this medium, arranged according to the length of incubation.

The influence of phosphate, of nitrate and of increased dextrose percentage is shown in table II. The medium contained 1.00 per

¹⁴ Travers: *Study of Gases*, 1901, p. 28.

¹⁵ Keyes: *Journ. of Med. Res.*, xxi (N. S. xvi), p. 69, 1909.

¹⁶ Dolt: *Journ. Inf. Dis.*, v, p. 616, 1908.

TABLE I.
B. coli in *vacuo*.

EXP. NO.	VOLUME OF MEDIUM	INCUBATION AT 37°	TOTAL GAS	CO ₂	H ₂	CO ₂ PER GRAM DEXTROSE	CO ₂ H ₂
	cc.	hours	cc.	per cent	per cent	cc.	
2	100	23.3	27.60	51.1		14.10	
3	25	40.0	7.21	49.5	48.1	14.30	1.03
4	100	44.3	28.40	51.3	48.1	14.55	1.06
5	100	58.2	28.60	51.1	47.0	14.60	1.09

cent of ammonium lactate and various quantities of dextrose, sodium phosphate and ammonium nitrate, as noted. In experiments 7, 8 and 9 Merck's "Highest Purity" dextrose was used; in experiment 6, the same recrystallized from alcohol; in experiment 10, a nitrate-free dextrose (Merck's "Pure") recrystallized.

The effect of an increase of phosphate is to increase the total gas formation, probably by delaying acid inhibition, and to increase the formation of carbon dioxide more than hydrogen.

The effect of nitrate is to decrease slightly the carbon dioxide formation and to use up most (or all) of the hydrogen that would otherwise be produced. The latter effect has been noted by Pakes and Jollyman.¹⁷ Merck's "Highest Purity" dextrose was found by the phenolsulphonic acid method to contain 0.04 per cent of sodium nitrate, which could be reduced to 0.01 per cent by recrystallization. Assuming proportionality, the result of experiment 9 would indicate that the amount of nitrate present in 1 gram of the "Highest Purity" dextrose could use up 0.012 cc. of hydrogen, a quantity which is negligible for *B. coli* fermentations but which would be of great significance in *B. typhosus* fermentations. The ammonium lactate and sodium phosphate were found free from nitrates by the same (controlled) test.

In another part of our work, to be communicated in a second paper, we have studied somewhat fully the effect of oxygen upon *B. coli* fermentations of dextrose in an asparagine medium. The following figures show the results for an aërobic fermentation by *B. coli* of a medium composed of 1.00 per cent "Highest Purity"

¹⁷ Pakes and Jollyman: *Journ. Chem. Soc. (Transactions)*, lxxix, p. 386, 1901.

TABLE II.
B. coli in *vacuo*.

EXP. NO.	COMPOSITION OF MEDIUM*			VOLUME OF MEDIUM cc.	INCUBATION AT 37° hours	TOTAL GAS cc.	CO ₂ per cent	H ₂ per cent	CO ₂ PER GRAM DEXTROSE cc.	CO ₂ / H ₂
	Dextrose		(NH ₄)NO ₃ per cent							
	per cent	N ₂ HPO ₄ per cent								
6	1.00	0.10		25	42.8	3.72	48.3	41.9	7.20	1.15
7	1.00	0.60		100	48.2	36.4	63.5		23.10	
8	1.00	0.60		100	52.7	68.3	63.5		43.40	
9	1.00	0.20	0.40	100	72.0	14.0	95.8		13.40	
10	4.00	0.20		50	580	20.4	50.3	47.2	5.12	1.07

* Exclusive of 1.00 per cent ammonium lactate.

TABLE III.
Gas production of B. typhosus in vacuo.*

EXP. NO.	VOLUME OF MEDIUM cc.	INCUBATION AT 37° hours	TOTAL GAS cc.	CO ₂ per cent	H ₂ per cent	RESIDUAL GAS per cent	SUM† per cent	PER GRAM OF DEX- TROSE		CO ₂ / H ₂
								CO ₂	H ₂	
								cc.	cc.	
1	213	48.2	3.26	92.4	2.4	5.1	99.9	1.41	0.038	38.5
2	193	72.2	3.40	92.10	1.96	5.98	100.04	1.63	0.033	47.0
3	250	360	5.44	97.10	2.13	0.76	99.99	2.12	0.044	45.6
4	150	384	3.22	93.25	2.03	4.44	99.72	1.99	0.042	46.0
5	150	108	2.01	92.15	4.90	2.47	99.52	1.24	0.066	18.8

* In experiments 1, 2 and 5 strain "A" was used; in experiments 3 and 4, strain "B."
† The figures in this column represent the sums of the analytical percentages recorded in the three preceding columns.

dextrose, 1.00 per cent ammonium lactate and 0.100 per cent disodium phosphate:

Experiment 11. B. Coli.

Oxygen admitted.....	4.59 cc.
Incubation at 37°.....	108. hours.
Total gas.....	10.56 cc.
CO ₂	5.18 cc.
O ₂	2.58 cc.
H ₂	0.96 cc.
CO ₂ per gram of dextrose.....	10.36 cc.
Ratio, CO ₂ :H ₂	5.4
Oxygen retained.....	2.01 cc.

Experiments 3, 4, 5 and 10 show that it is possible to find conditions such that nearly equal volumes of CO₂ and H₂ are obtained but that the CO₂ is always in slight excess. Evidently the presence of oxygen, of nitrates or of sodium phosphate, since all these substances tend to increase the ratio of carbon dioxide to hydrogen, prevents the realization of the value unity for this ratio. Dolt¹⁸ found that *B. coli* required either phosphates or nitrates for its growth. We have found that in the absence of nitrates anaërobic growth vanishes if the phosphate content is sensibly reduced below the lowest concentration used in the experiments given above, so that it appears that, in spite of a systematic error due to the greater solubility of carbon dioxide in the culture liquid, it is not possible to collect equal volumes of carbon dioxide and hydrogen. This means that if, as Harden concluded, the decomposition of dextrose by the action of *B. coli* results in the formation of an equal number of molecules of carbon dioxide and hydrogen, according to the "classical" fermentation of Duclaux,¹⁹ there occurs also a process which either uses up hydrogen or produces carbon dioxide and which is therefore oxidational. This process seems to be necessary for growth.

Experiments with B. typhosus.

A few experiments were made with two strains of the typhoid bacillus. Neither strain could be grown on Uschinski's solution, on Fraenkel's modification or on various other simple media.

¹⁸ Dolt: *Journ. Inf. Dis.*, v, p. 616, 1908.

¹⁹ Duclaux: *Traité de microbiologie*, iv, p. 49, 1901.

The culture fluid consisted of 1.00 per cent each of Witte's peptone and dextrose in distilled water, prepared hot and filtered. In the first four experiments Merck's "Highest Purity" dextrose was used and in the fifth a preparation made by recrystallizing Merck's "Pure" (nitrate-free) dextrose from alcohol.

The following analysis for experiment 5 typifies the procedure followed in the analysis of small quantities of gases. The expo-

Analysis of experiment 5.

	OBSERVED VOLUME	MANOMETER READINGS		PRESSURE	TEMPER- ATURE	VOLUME AT 760 MM.	MEAN VOLUME
					°C.		
Total gas...	{ 4.20 6.80 5.70	523 357 413	124 114 118	399 243 295	22.0 22.9	2.150 2.180 2.220	2.183
After KOH. . .	{ 1.00 5.00 2.35 1.80 1.40	264 147 187 204 234	138 121 132 134 135	126 26 55 70 99	22.5 23.0 22.9	0.166 0.171 0.170 0.166 0.182	0.171
Air added . . .	{ 2.60 3.75 5.15 5.80 4.60	544 425 339 309 365	130 126 120 118 123	414 299 219 191 242	23.1 23.0	1.420 1.470 1.480 1.460 1.460	1.458
Pass spark...	4.60	363	123	244		1.460	
Added elec- trolytic gas in small quantities until mix- ture ex- ploded on passing spark.	{ 3.20 5.60 6.80 3.40	442 296 258 410	129 119 114 128	313 177 144 282	 23.0	1.310 1.300 1.290 1.290	1.298

The result (volumes at room temperature, but dry without correction because the barometer space was saturated with moisture) is therefore

CO ₂	2.012	Residual.....	0.054
H ₂	0.107	Sum.....	2.173

All gas volumes were further reduced to 0°C.

sive gas was calculated as hydrogen, and the closeness with which the sums of the analytical percentages thus found approach 100 per cent indicates that the explosive gas was hydrogen. On so small a quantity of gas as that remaining for the hydrogen determination the non-production of carbon dioxide upon explosion could not be certainly proved.

Since the presence of dextrose is of great importance for growth of bacteria in the absence of oxygen, it is not possible to show by the omission of dextrose what is probably the truth: that the gases obtained came from the dextrose. Nevertheless a significant amount of fermentable sugar could not have been present in the peptone, since it was found that *B. coli* produced anaerobically on a 1 per cent solution of Witte's peptone in forty-eight hours at 37°C. only 0.095 cc. of CO₂ (38 per cent of the total gas) per gram of peptone, and *B. typhosus* produced under the same conditions less than 0.02 cc. of CO₂ per gram of peptone.

These results show that carbon dioxide and hydrogen are evolved by the action of *B. typhosus* on dextrose, but that the amounts produced are very much less than those produced by *B. coli*, and the ratio of carbon dioxide to hydrogen is many times higher in the case of *B. typhosus*. Upon the nitrate-free dextrose (experiment 5) considerably more hydrogen is produced. The increase per gram of dextrose is about 0.027 cc., whereas the volume of hydrogen which the amount of nitrate in question can use up in a *B. coli* fermentation we have found above to be 0.012 cc.

Just as the amount of nitrate present as impurity in the dextrose, while it introduced no significant error in the results for *B. coli*, made a large difference in the results for *B. typhosus* (where the total amount of gas was much smaller), so may the principles in the peptone which increase the ratio with *B. coli* make a greater difference with *B. typhosus*. It is possible that this is wholly responsible for the difference in the values of the ratio found for the two organisms.

Experiments with Bact. welchii.

Bact. welchii was grown anaerobically on a medium consisting of 1 per cent each of Witte's peptone and Merck's "Highest Purity" dextrose in distilled water. No alkali was added.

TABLE IV.

Bact. welchii in vacuo.

EXP. NO.	VOLUME OF MEDIUM	INCUBATION AT 37°	TOTAL GAS	CO ₂	H ₂	RESIDUAL GAS	CO ₂ PER GRAM DEXTROSE	CO ₂ H ₂
	cc.	hours	cc.	per cent	per cent	per cent	cc.	
1	100	120	61.3	60.0	39.3	0.5	36.8	1.53
2	50	336	63.5	58.5	41.2	0.3	74.2	1.43

The results differ chiefly from those found for *B. coli* upon this medium in the larger volumes of gas found per gram of dextrose.

Comparison of the three microorganisms.

Some of the foregoing experiments give a basis for a comparison of the three organisms, namely, those anaërobic fermentations of a medium consisting of 1 per cent each of Witte's peptone and dextrose. In table V are given the maximum volumes of carbon dioxide evolved per gram of dextrose and the ratio CO₂:H₂. The lower value of the ratio given under *B. typhosus* is that obtained with nitrate-free dextrose.

TABLE V.

	<i>B. TYPHOSUS</i>	<i>B. COLI</i>	<i>BACT. WELCHII</i>
Carbon dioxide in cubic centimeters	2.1	30.7*	74.
Ratio, CO ₂ :H ₂	from 19 to 44	1.31	1.48

*48.2 hours; possibly not maximum amount obtainable.

SUMMARY.

The gas evolution accompanying the growth of certain bacteria on culture media containing dextrose has been studied by an exact method.

I. The principal results for *B. coli* are:

A. Dextrose-peptone media yield considerably larger volumes of carbon dioxide than of hydrogen upon anaërobic fermentation. The volume-ratio CO₂:H₂ is 1.31.

B. A suitable "synthetic" medium (composed of ammonium lactate, disodium phosphate and dextrose) yields anaërobically nearly equal volumes of the two gases. The ratio CO₂:H₂ is always

greater than unity and has a mean value of 1.06 for a medium of given composition.

1. The presence of oxygen raises the value of this ratio.

2. Increase of phosphate content also raises the value.

3. The phosphate cannot be reduced sensibly in quantity, or substituted by a salt less objectionable.

4. The value 1.06 for the ratio $\text{CO}_2:\text{H}_2$ is minimal. This means that if the principal gas reaction consists of a liberation of an equal number of molecules of carbon dioxide and hydrogen from dextrose there also occurs an accompanying gas reaction of the nature of an oxidation.

II. *B. typhosus* produces anaerobically from a dextrose-peptone medium small volumes of carbon dioxide and an explosive gas, probably hydrogen. The ratio $\text{CO}_2:\text{H}_2$ is never lower than 19.

III. *Bact. welchii* produces anaerobically from a dextrose-peptone medium large volumes of carbon dioxide and hydrogen. The ratio $\text{CO}_2:\text{H}_2$ is 1.48.



A CONTRIBUTION TO OUR KNOWLEDGE OF THE GAS METABOLISM OF BACTERIA.

SECOND PAPER.

THE ABSORPTION OF OXYGEN BY GROWING CULTURES OF *B. COLI* AND OF *BACT. WELCHII*.

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(*From the Biological Laboratory of Brown University.*)

(Received for publication, October 9, 1912.)

We have studied the gas production of *B. coli* and of *Bact. welchii* for various incubation periods in the presence of oxygen admitted to the fermentation bulbs in known quantities after a vacuum had first been obtained. The technique employed has been described by us in the preceding paper.

From the data so obtained we can derive information concerning the rate at which oxygen is absorbed and concerning the relations existing among the quantities of oxygen absorbed and the quantities of carbon dioxide and hydrogen evolved.

We have not found in the literature any work on these points for any microorganism where the atmosphere over the culture was accurately controlled and the analyses were made on portions of gas accurately sampled.

Experiments with B. coli.

The culture medium consisted of 1.00 per cent each of Merck's "Highest Purity" dextrose and asparagine and 0.200 per cent of disodium phosphate and was made neutral to phenolphthalein with sodium hydrate. Corrections were made for the carbon dioxide thus introduced, as explained in the first paper, and the values of the corrections are given with the analyses.

The results of the gas analyses are given in table I.

The values of the ratio, $\text{CO}_2:\text{H}_2$, vary enormously, and are many times the value previously obtained by one of us¹ for anaërobic fermentations.

In an experiment described in the preceding paper (Exp. 11, p. 299) the retention of oxygen was found to be 2.0 cc., whereas the decrease in yield of hydrogen (*i.e.*, that amount which, together with the amount actually found, would make the ratio $\text{CO}_2:\text{H}_2$ equal to 1.06, the mean value for anaërobic fermentations on the same medium) is 3.9 cc. or only 0.1 cc. less than that which the oxygen could have oxidized. The medium for which this result was obtained was based on ammonium lactate. If this result were the rule, it would indicate that the oxygen was almost quantitatively taken up by nascent hydrogen from the dextrose. Similar calculations from the data here presented show, however, that this is not the rule (at least in the case of the asparagine medium) but that the missing volumes of hydrogen are sometimes greater and sometimes smaller than twice the volumes of absorbed oxygen.

The volume of oxygen absorbed per unit volume of carbon dioxide evolved (the "respiratory quotient") is given. The mean value is 0.135, with a probable error of ± 0.02 .

The relation between the volume of oxygen admitted to the culture, the volume of oxygen not absorbed and the duration of incubation at 37°C. can well be seen from a calculation of the values of the expression $\frac{1}{t} \cdot \log \frac{V}{v}$; where t is the time in hours, V is the volume of oxygen admitted, and v is the volume of the unabsorbed oxygen. Since $\frac{V}{v}$ here equals the ratio of the corresponding pressures, the given expression is that for the constant of a monomolecular gas reaction. It is in fact nearly constant. The values are given in table III. We should note, however, that the fermentation bulbs were not shaken during incubation, so that although a certain degree of agitation was imparted to the medium by the brisk evolution of gas, the rate of oxygen absorption may, nevertheless (namely, if relatively fast), have been limited by the rate of distribution. In this case the given expression should be constant.

¹ Keyes: *Journ. of Med. Res.*, xxi (N.S., xvi), p. 69, 1909.

The data given in table I may properly be compared with those obtained for anaërobic fermentations induced by *B. coli* on the same medium, and given by one of us in a previous paper,² with the following results: (1) Smaller volumes of carbon dioxide are produced aërobically than anaërobically, for all periods of time. (2) For the same amount of carbon dioxide, less hydrogen is obtained aërobically than anaërobically. The presence of oxygen therefore appears to lessen the production of gases from dextrose and also either to cause some output of carbon dioxide by a respiratory process or to cause a disappearance of hydrogen (presumably) by oxidation.

Experiments with Bact. welchii.

Similar experiments were made with *Bact. welchii*. The medium consisted of 1.00 per cent each of Witte's peptone and Merck's "Highest Purity" dextrose in distilled water, prepared hot and filtered. No alkali was added. Other conditions were the same as before, except that all durations of incubation were much longer and the pressures of oxygen were much smaller.

The values of the expression $\frac{1}{t} \log \frac{V}{v}$ are given in table III. They are very nearly constant and are about one-third the value found for *B. coli*.

All other results are given in table II. The mean value for the respiratory quotient $\left(\frac{O_2}{CO_2}\right)$ is 0.014, i.e., one-tenth the value found for *B. coli*, with a probable error of ± 0.002 .

The ratio $CO_2:H_2$ is slightly raised by the presence of oxygen,³ and does not vary for this organism in great degree as it does for *B. coli*.

As with *B. coli*, the "missing" volumes of hydrogen are not equal to twice the volumes of oxygen absorbed but are sometimes greater and sometimes smaller. It is perhaps of significance in this connection that both the media used for these organisms permit side-reactions which prevent the evolution of equal volumes of carbon dioxide and hydrogen by anaërobic fermentation of dextrose, as discussed in the first paper of this study.

² Keyes: *loc. cit.*

³ Values for anaërobic fermentations are given in the first paper of this series.

TABLE I.
B. coli.

All gas volumes are reduced to standard conditions: 0°C., 760 mm. of mercury and to dryness.

WEIGHT	WEIGHT MEDIA	CORRECTION FOR CO ₂ IN NaOH	SPACE ABOVE LIQUID	O ₂ ABSORBED	INITIAL PRESSURE OF O ₂ IN MM. Hg. (APPROX.)	INCUBATION AT 37°C.	TOTAL GAS FOUND	CO ₂ FOUND	CO ₂ CORR. FOR NaOH	O ₂ FOUND	H ₂ FOUND	N ₂ FOUND	VOLUME-RATIO, $\frac{H_2}{CO_2}$	O ₂ ABSORBED	RESPIR. QUOTIENT $\frac{O_2}{CO_2}$	CO ₂ PER GRAM DEXTROSE	CO ₂ PER GRAM ANAEROBICALLY (EXPERIMENTS OF KEYS, '09)
1	2.00	0.78	50	5.27	80	24	28.61	24.52	23.74	3.81	0.04	0.24	590.0	1.46	0.062	11.87	16.9
2	1.35	0.60	115	5.34	35	46	20.06	14.79	14.79	3.40	1.74	N.D.*	8.6	1.94	0.132	10.95	29. - 63.
3	2.41	0.88	154	5.28	26	72	36.52	25.75	24.87	2.74	7.38	0.75	3.4	2.54	0.102	10.32	29. - 63.
4	1.12	0.46	183	10.36	43	74	29.82	19.65	19.19	5.35	4.52	0.30	4.2	5.01	0.261	17.12	29. - 63.
5	2.00	0.78	50	5.05	76	119	37.47	34.11	33.33	2.68	0.17	0.52	196.0	2.37	0.071	16.67	63.4+
6	1.80	0.57	70	7.38	80	167	48.97	45.94	45.37	2.29	0.47	0.33	96.5	5.09	0.112	25.20	63.4+
7	2.40	0.85	55	5.97	82	192	27.07	21.33	20.48	1.79	2.44	1.44	8.4	4.18	0.204	8.36	63.4+
															Mean		
															= 0.135		
															± 0.02		

* In anaerobic fermentations this ratio does not change with the time and has the mean value 1.77 (Keys: *Journ. Amer. Chem. Soc.*, xxxi, p. 1271).

† N. D. = Not done.

TABLE II.
Bact. welchii.

All gas volumes are reduced to standard conditions.

NUMBER	WEIGHT MEDIUM grams	SPACE ABOVE LIQUID cc.	O ₂ ADDED cc.	INITIAL PRESSURE of O ₂ in MM. Hg. (APPROX.)	INCUBATION AT 37°C. hours	TOTAL GAS FOUND cc.	CO ₂ FOUND cc.	O ₂ FOUND cc.	H ₂ FOUND cc.	N ₂ FOUND cc.	VOLUME-RATIO, CO ₂ :H ₂	O ₂ ABSORBED cc.	RESPIR. QUOTIENT CO ₂ /O ₂	CO ₂ PER GRAM DEXTROSE cc.	CO ₂ PER GRAM ANABOLICALLY (FIRST PAPER OF THIS STUDY) cc.
1	63.0	237	0.38	0.12	240	73.28	43.35	0.18	28.38	0.42	1.53	0.19	0.0044	68.80	
2	57.5	242	0.97	3.05	240	57.07	36.06	0.55	19.55	0.90	1.85	0.42	0.0116	62.80	
3	42.5	253	0.94	2.85	336	54.36	33.20	0.65	19.84	0.61	1.67	0.29	0.0088	78.20	
4	52.0	248	0.73	2.25	384	65.14	38.20	0.28	26.53	N.D.†	1.44	0.45	0.0118	73.50	
5	55.0	245	1.24	3.85	432	71.92	45.01	0.28	25.72	0.72	1.75	0.96	0.0214	81.90	
6	49.0	251	1.62	3.90	480	71.55	45.45	0.37	25.20	0.55	1.80	1.25	0.0275	92.80	
													Mean = 0.014 ± 0.002		74.20

* For anaerobic fermentations the values for this ratio were 1.53 and 1.42, as found in the preceding paper.

† N.D.—Not done.

TABLE III.

Values of $\frac{1}{t} \log \frac{\text{volume O}_2 \text{ admitted}}{\text{volume O}_2 \text{ recovered}}$

t = time in hours; common logarithms.

HOURS	<i>B. COLI</i>	HOURS	<i>BACT. WELCHII</i>
24	0.0059	240	0.0013
66	0.0030	240	0.0010
72	0.0040	336	0.0005
74	0.0039	384	0.0011
119	0.0023	432	0.0015
167	0.0030	480	0.0013
192	0.0027		
Mean value	0.0035 \pm 0.0003		0.0011 \pm 0.0001

SUMMARY.

The absorption of oxygen by growing cultures on dextrose media of *B. coli* and of (the strict anaërobe) *Bact. welchii* has been studied by an exact method. Data afforded by complete gas analyses are given. The following comparisons are made possible:

1. For both microorganisms, the absorption of oxygen simulates a monomolecular reaction.

2. The mean values of the respiratory quotients, although the probable error of each is large, are widely different for the two microorganisms.

3. With varying pressures of oxygen, the ratio $\text{CO}_2:\text{H}_2$ varies enormously in the case of *B. coli*, but varies only slightly in the case of *Bact. welchii*.

We think that the numerical differences in the results for the two microorganisms may possibly be referable to differences in experimental conditions; if so, probably to the differences in oxygen pressures.

THE ORGANIC PHOSPHORIC ACID OF COTTON SEED MEAL.

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INTRODUCTION.

In the investigation of the organic phosphoric acids present in various cereals and feeding stuffs which is being carried out in this laboratory, cotton seed meal was also examined. Earlier work by other investigators has shown that this product probably contains some complex organic phosphoric acid.¹ It seems, however, that if such a substance is present it has not been isolated in pure form nor have its properties been fully studied.

The opinion seems to be generally held that cotton seed meal contains some poisonous principle, but the exact nature of this principle has never been definitely determined. It has been claimed that pyro- and metaphosphoric acids were present in cotton seed meal² and it was thought that the poisonous properties of the product were due to the presence of salts of these acids. More recent work by Crawford³ led him to believe that the poisonous principle was a salt of either a simple inorganic or a complex organic pyrophosphoric acid.

The presence of these acids has been adduced from the fact that extracts of cotton seed meal give similar reactions to the above acids, viz., anomalous behavior towards ammonium molybdate, white precipitates with silver nitrate and coagulation

¹ Rather: *Texas Agric. Exp. Station, Bulletin 146*, 1912.

² Hardin: *South Carolina Agric. Exp. Station, Bulletin 8* (new series), 1892.

³ Crawford: *Journ. of Pharmacol. and Exp. Therapeutics*, i, p. 519, 1910.

of egg albumen; further, the poisonous effects resemble those given by these acids. Aside from these reactions, however, there is no proof whatever that either pyro- or metaphosphoric acid is present in cotton seed meal.

The purpose of the present investigation was to isolate and identify, if possible, the organic phosphoric acid in cotton seed meal. We are, consequently, unable either to deny or affirm the absence or presence of pyro- or metaphosphoric acid in this product. We have found, however, that the organic phosphoric acid isolated from cotton seed meal gives all the reactions reported by the above authors, which they considered as evidence of the presence of pyro- and metaphosphoric acid. It seems, therefore, probable that the reactions referred to are due to the organic phosphoric acid rather than to pyro- or metaphosphoric acids.

The preparation of the substance and its purification will be more fully described in the experimental part. It will suffice to state here that the cotton seed meal was extracted with 0.2 per cent hydrochloric acid and the substance isolated as the barium salt. The purification of the substance is very difficult. The extract contains large quantities of soluble impurities, mucilaginous substances, proteins, etc., which render the purification extremely difficult and tedious. In addition to the above, there is apparently some carbohydrate associated with the organic phosphoric acid, the removal of which requires much time. For the same reasons the yield of the pure product is very unsatisfactory.

The compound finally obtained is very similar to phytic acid so far as composition and reactions are concerned. In fact it is impossible to determine from the present data whether the substance is phytic acid or an isomer. Both yield inosite when heated in a sealed tube with dilute sulphuric acid and the reactions of aqueous solutions of the free acids can hardly be differentiated. The most striking difference is that the barium salt of the product from cotton seed meal shows a decided tendency to crystallize, a property which we have never observed when working with barium phytate under the same conditions.

If the substance from cotton seed meal is precipitated from acid solutions with barium hydroxide it separates as a white

amorphous precipitate. When the dried precipitate is digested in 0.5 per cent hydrochloric acid it dissolves very readily but after a few minutes it precipitates again. Under the microscope this precipitate is seen to consist of balls or globular masses of very fine needle-shaped crystals. The dilute hydrochloric acid solution of the barium salt gives a white amorphous precipitate on the addition of alcohol; on standing for several hours, however, it slowly assumes the same crystalline form as mentioned above. The free acid is not precipitated by barium chloride but if such a solution is allowed to stand over night or longer the barium salt will separate in fine needle-shaped crystals, grouped in the same general form as above but the individual crystals are much larger. The amorphous precipitates are very soluble in 0.5 per cent hydrochloric acid but, after the substance has assumed the crystalline form, it is very slightly soluble in this medium.

While the barium salt was easily obtained in crystalline form it did not contain a constant amount of the base. The variations would sometimes amount to as much as 3 or 4 per cent, depending upon the amount of the base present in the solution and the conditions under which the substance separated. In the presence of a large excess of barium chloride a salt corresponding nearly to tetrabarium phytate crystallizes out; when a small amount of barium chloride is present salts showing the above mentioned variations are formed; but when the substance has been repeatedly separated from acid solutions with alcohol a salt is obtained which corresponds nearly to tribarium phytate.

The aqueous solution of the free acid gives a heavy white amorphous precipitate with excess of silver nitrate; with ammonium molybdate a heavy white crystalline precipitate is produced which remains unchanged in the cold for a long time but when heated soon turns yellowish in color. These reactions are identical with those given by phytic acid; with other metals both acids give apparently identical reactions.

The dilute aqueous solution of the acid coagulates egg albumen at once. This property of coagulating egg albumen, however, is not peculiar to the acid from cotton seed meal. Phytic acid was found to produce an identical effect. The tetraphosphoric acid

ester of inosite⁴ and the pyrophosphoric acid esters of inosite⁴ mentioned in former papers also gave the same reaction. In view of the fact that the last mentioned substances coagulate egg albumen, it appears probable that this property is common to organically bound phosphoric acids.

As will be noticed from the foregoing the organic phosphoric acid of cotton seed meal gives all the reactions previously attributed to the presence of pyro- and metaphosphoric acids. But the question whether or not it is also the toxic principle in cotton seed meal remains unanswered. Preliminary experiments carried out with the acid obtained from the purified barium salt on rabbits are not conclusive. Given in 0.5 and 1 gram doses, the free acid or its potassium salt produced strong symptoms of distress but after a few hours the animals regained their normal appearance. Larger doses passed through the bowel in a very short time and no definite symptoms developed.

It is difficult to determine just what caused the toxicity of the preparations which were used in the experiments described by Crawford.⁶ It is evident that very impure substances were given.

It is our purpose to carry out a series of experiments to determine the toxicity of the acid from cotton seed meal in comparison with phytic acid.

EXPERIMENTAL.

The cotton seed meal used in these experiments was obtained from the stock used as cattle feed in this institution. For the first preparation 4500 grams of meal were digested in 10 liters of 0.2 per cent hydrochloric acid over night. It was then pressed through cheese-cloth and the extract filtered through a layer of clean sand. The extract was a thick, mucilaginous, very dirty colored liquid which could not be filtered through paper. It measured about 5 liters. It was mixed with about 8 liters of alcohol which produced a very fine and voluminous dirty pre-

⁴ Anderson: this *Journal*, xi, p. 484, 1912; Tech. Bulletin 19, N. Y. Agric. Exp. Station, 1912.

⁵ Anderson: this *Journal*, xii, pp. 109, 111, 1912; Tech. Bulletin 21, N. Y. Agric. Exp. Station, 1912.

⁶ *Loc. cit.*

cipitate. After settling over night the supernatant liquid was syphoned off and the residue centrifuged. The precipitate was then digested in a considerable quantity of 0.5 per cent hydrochloric acid, the insoluble portion removed by centrifuging and the solution precipitated with excess of barium hydroxide. The mixture was heated nearly to boiling and then allowed to cool and settle. It was again centrifuged and the residue treated with 0.5 per cent hydrochloric acid in which it was readily soluble. After a few minutes, however, it began to separate as a fine crystalline precipitate. The mixture was then filtered and the above precipitate reserved for special examination.

The filtrate was precipitated by the addition of alcohol, filtered, again treated with 0.5 per cent hydrochloric acid, filtered from insoluble matter and again precipitated by alcohol. It was filtered and washed in dilute alcohol and then dissolved in 0.5 per cent hydrochloric acid, heated nearly to boiling and filtered. The filtrate was now nearly colorless and it was slightly opalescent in appearance. After again precipitating the hydrochloric acid solution with alcohol the substance was obtained as a white amorphous powder. It was very soluble in 0.5 per cent hydrochloric acid but the solution had a thick, mucilaginous and slightly, opalescent appearance. This solution was now precipitated with excess of barium hydroxide when a voluminous, tenacious, ropy precipitate was obtained. The mixture was thoroughly shaken for some time and then filtered and washed in water. The washed residue was dissolved in 0.5 per cent hydrochloric acid and precipitated with alcohol. After repeating this operation the substance was filtered, washed free of chlorides with dilute alcohol and finally washed in alcohol and ether and dried in vacuum over sulphuric acid. The product was then a snow-white amorphous powder, weighing 10.2 grams.

The substance thus prepared was but slightly soluble in boiling water. With phloroglucine and hydrochloric acid it gives a light red color which soon changes to a reddish-brown. With orceine it gives at first a reddish color which soon fades, leaving a dirty colored precipitate. After boiling the substance in dilute hydrochloric acid, precipitating the barium with sulphuric acid, filtering and neutralizing, it strongly reduces Fehling's solution on boiling. The nitric acid solution gave no reaction with ammo-

nium molybdate but after continued heating a slight precipitate was obtained. The substance was free from nitrogen and sulphur.

After drying at 105° in vacuum over phosphorus pentoxide it was analyzed.

0.2925 gram substance gave 0.0894 gram H_2O and 0.2338 gram CO_2 .

0.2514 gram substance gave 0.0972 gram $BaSO_4$ and 0.0933 gram $Mg_2P_2O_7$.

Found: C = 21.80; H = 3.42; P = 10.34; Ba = 22.75 per cent.

While the substance was very slightly soluble in boiling water it was found when it was rubbed up in a mortar with a small quantity of cold water that it quickly dissolved but it began soon to separate again. Under the microscope the precipitate was seen to consist of small balls or globular masses of very fine microscopic needles.

Four grams of the substance were treated as mentioned above. After standing for two days at room temperature the crystalline precipitate was filtered off, washed in water, alcohol and ether and dried in the air. The snow-white crystalline powder was analyzed after previous drying at 105° in vacuum over phosphorus pentoxide.

0.2092 gram substance lost 0.0291 gram H_2O .

0.1801 gram substance gave 0.0232 gram H_2O and 0.0379 gram CO_2 .

0.1754 gram substance gave 0.1262 gram $BaSO_4$ and 0.0965 gram $Mg_2P_2O_7$.

Found: C = 5.73; H = 1.44; P = 15.33; Ba = 42.34; H_2O = 13.91 per cent.

The composition of this product differs entirely from that of the starting material but it agrees closely with that required for tetrabarium phytate.

Calculated for tetrabarium phytate, $C_6H_{16}O_{27}P_6Ba_4$ = 1255:

C = 5.73; H = 1.27; P = 14.82; Ba = 43.74; 11 H_2O = 13.62 per cent.

The filtrate from the above crystalline compound was precipitated by alcohol, filtered, washed and dried in vacuum over sulphuric acid. It was a perfectly white amorphous powder. It was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.1758 gram substance gave 0.0790 gram H_2O and 0.2222 gram CO_2 .

0.1247 gram substance gave 0.0223 gram $BaSO_4$ and 0.0201 gram $Mg_2P_2O_7$.

Found: C = 34.47; H = 5.02; P = 4.49; Ba = 10.52 per cent.

The substance was very soluble in 0.5 per cent hydrochloric

acid, in which it gave the same thick, mucilaginous, slightly opalescent solution as mentioned above.

The compound first analyzed is evidently not homogeneous. It apparently consists of some carbohydrate or gummy substance and an organic phosphorus compound; the latter crystallizes from the aqueous solution in nearly pure form but the substances cannot be separated by precipitating the dilute acid solutions with alcohol. This gummy substance has not been isolated in pure form and we are entirely in the dark as to its nature and composition.

A portion of the above crystalline barium salt was used for the preparation of the free acid. The substance was, however, not pure and it had not been washed free of the mother liquor. The acid was prepared by the usual method, that is, the barium salt was decomposed with a slight excess of sulphuric acid, the filtered solution precipitated with copper acetate, the latter filtered, washed and decomposed with hydrogen sulphide, again filtered and the filtrate evaporated in vacuum at a temperature of 40–45° and finally dried in vacuum over sulphuric acid. In appearance and reactions the acid was practically identical with phytic acid except that after boiling with dilute hydrochloric acid and neutralizing it slightly reduced Fehling's solution. This reduction, however, we believe to be due to admixed impurities for, as stated above, the acid was not prepared from a pure compound.

The aqueous solution of the acid coagulates egg albumen at once. As has been already mentioned phytic acid gives the same reaction, as well as the inosite esters of phosphoric and pyrophosphoric acids. Apparently, therefore, no special significance can be attached to this reaction.

The acid gave the following result on analysis after previously drying at 105° in vacuum over phosphorus pentoxide.

Found: C = 11.71; H = 3.07; P = 26.35 per cent.

The crystalline precipitate mentioned on page 315, which separated from the solution of the first barium precipitate in 0.5 per cent hydrochloric acid, was treated with about 5 per cent hydrochloric acid in which the greater portion dissolved. The insoluble matter was removed by centrifuging and the solution precipitated with alcohol. This operation was repeated a second time when

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the substance was obtained nearly white. It differed from the first preparation in that its solution in dilute hydrochloric acid was neither mucilaginous nor opalescent. For further purification the substance was first precipitated by barium hydroxide from its dilute hydrochloric acid solution and then twice precipitated from dilute hydrochloric acid with alcohol. The precipitates produced by the alcohol were amorphous at first but when allowed to stand over night in the mother liquor they always changed to the same crystalline form as previously mentioned.

After precipitating the last time with alcohol the substance was quickly filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a snow-white amorphous powder and it weighed 7.4 grams.

The filtrate from the above was allowed to stand over night when a small amount of the substance crystallized out. The crystals were filtered, washed in dilute alcohol, alcohol and ether and dried in the air. The substance was free from chlorine and gave no appreciable color reaction with phloroglucine or orcin. For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

0.2450 gram substance lost 0.0272 gram H₂O on drying.

0.2178 gram substance gave 0.0284 gram H₂O and 0.0522 gram CO₂.

0.1776 gram substance gave 0.1170 gram BaSO₄ and 0.1078 gram Mg₂P₂O₇.
Found: C = 6.53; H = 1.45; P = 16.91; Ba = 38.76; H₂O = 11.10 per cent.

This substance agrees nearly in composition with tribarium phytate.

Calculated for tribarium phytate, C₆H₁₈O₂₇P₃Ba₃ = 1120:

C = 6.42; H = 1.60; P = 16.60; Ba = 36.78; 8H₂O = 11.39 per cent.

The amorphous product (7.4 grams) mentioned above was analyzed after previous drying at 105° in vacuum over phosphorus pentoxide and the following results obtained:

C = 8.04; H = 1.62; P = 16.65; Ba = 36.55 per cent.

The substance was free from chlorine. It was very slightly soluble in boiling water. With phloroglucine it gave a cherry red color; with orcin only a faintly greenish color was produced. After boiling in dilute hydrochloric acid, precipitating the barium with sulphuric acid, filtering and neutralizing, it reduced Fehling's solution slightly on boiling. Evidently some carbohydrate was still present.

For further purification the substance was dissolved in 0.5 per cent hydrochloric acid, filtered and alcohol added until a faint permanent turbidity remained. This was cleared up by the addition of a few drops of dilute hydrochloric acid and the solution allowed to stand at room temperature. The substance soon began to separate in the same crystalline form as before. After standing for two days the crystalline substance was filtered off, washed in water, alcohol and ether and dried in vacuum over sulphuric acid.

The mother liquor was diluted with more alcohol and allowed to stand as before when a further quantity of the same shaped crystals was obtained. After filtering, washing and drying as before these salts were analyzed after first drying at 105°.

Found (first crop of crystals): C = 7.06; H = 1.53; P = 16.46; Ba = 38.16 per cent.

Found (second crop of crystals): C = 7.47; H = 1.58; P = 16.46; Ba = 38.12 per cent.

In order to determine if further treatment would alter the composition, the whole substance was digested in 50 per cent acetic acid over night, filtered, washed in water, alcohol and ether and dried in the desiccator. It was then dissolved in 0.5 per cent hydrochloric acid, filtered and the solution brought to crystallization by the careful addition of alcohol as before. The product finally obtained weighed 3.8 grams and it was a snow-white crystalline powder. For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

Found: C = 7.10; H = 1.52; P = 17.17; Ba = 38.11 per cent.

As continued treatment did not alter the composition and as it separated in crystalline form it was undoubtedly a homogeneous compound.

The free acid prepared from this purified barium salt by the same method as before gave the following result on analysis after previous drying at 78° in vacuum over phosphorus pentoxide:

0.2626 gram substance gave 0.0763 gram H_2O and 0.1049 gram CO_2 .

0.1733 gram substance gave 0.1686 gram $Mg_2P_2O_7$.

Found: C = 10.89; H = 3.25; P = 27.11 per cent.

Calculated for phytic acid, $C_8H_{24}O_{17}P_8$ = 714:

C = 10.08; H = 3.36; P = 26.05 per cent.

This acid gave the same reactions as previously described.

Preparation of inosite from the above barium salt.

Of the dry salt, 1.34 grams were heated in a sealed tube with 10 cc. of 5*N* sulphuric acid to 160° for about three hours. After precipitating with barium hydroxide the inosite was isolated in the usual way and recrystallized from dilute alcohol with addition of ether. The product was obtained in colorless needles free from water of crystallization. The yield was 0.17 gram or about 77 per cent of the theory. It gave the reaction of Scherer and melted at 221° (uncorrected). The air-dried substance was analyzed:

Found: C = 39.81; H = 6.96 per cent.

A further quantity of the barium salt was prepared by the following method which was found to be much less laborious than that used at first. The cotton seed meal, 8 kilograms, was digested in 16 liters of 0.2 per cent hydrochloric acid for about five hours. It was then pressed through cheese-cloth and the extract filtered through absorbent cotton. The extract was precipitated with excess of barium hydroxide, allowed to settle and then centrifuged. The precipitate was digested in several liters of 0.5 per cent hydrochloric acid and again centrifuged. The free acid was then nearly neutralized with barium hydroxide. The precipitate which separated was the barium salt of the organic phosphoric acid. This was filtered and treated with 0.5 per cent hydrochloric acid in which it was readily soluble at first but it soon separated in the usual crystal aggregates. This was filtered and washed and dissolved in sufficient dilute hydrochloric acid and again filtered. The practically colorless filtrate was precipitated by alcohol. After filtering it was again dissolved in dilute hydrochloric acid and precipitated with barium hydroxide, filtered and washed in water. It was then dissolved in dilute hydrochloric acid, precipitated with alcohol, filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a snow-white amorphous powder and it weighed 24 grams. It was dissolved in about 300 cc. of 0.5 per cent hydrochloric acid, filtered and allowed to stand a short time when a portion crystallized out. This was filtered off, washed several times in water and finally in alcohol and ether and dried in the air. The white crystalline powder weighed 7.4

grams. The filtrate and washings from above were united and precipitated by alcohol. After standing over night the amorphous precipitate had changed to the usual crystalline form. After filtering, washing and drying in vacuum over sulphuric acid it weighed 14.7 grams.

The above salts were free from chlorine. The nitric acid solutions gave no immediate reaction with ammonium molybdate. No appreciable color reactions were obtained with phloroglucine or orcin and they did not reduce Fehling's solution. Metals other than barium were absent. For analysis the substances were dried at 105° in vacuum over phosphorus pentoxide.

The first crystalline compound gave the following:

C = 6.05; H = 1.45; P = 16.51; Ba = 40.04; H_2O = 12.06 per cent.

This salt is evidently a mixture of the tri- and tetrabarium salt. It was recrystallized as follows: One gram of the substance was dissolved in about 150 cc. of 0.5 per cent hydrochloric acid and the free acid nearly neutralized with barium hydroxide. About 0.5 gram of barium chloride was then added and the solution allowed to stand for two days at room temperature. The substance separated slowly in the same general crystal form as before except that the individual crystals were much larger. These were filtered, washed in water, alcohol and ether and dried in the air. Under the microscope the substance appeared homogeneous. Yield, 0.9 gram. After drying at 105° in vacuum it was analyzed:

0.4067 gram substance lost 0.0496 gram on drying.

0.3571 gram substance gave 0.0358 gram H_2O and 0.0699 gram CO_2 .

0.2068 gram substance gave 0.1530 gram $BaSO_4$ and 0.1160 gram $Mg_2P_2O_7$.

Found: C = 5.33; H = 1.12; P = 15.63; Ba = 43.53; H_2O = 12.19 per cent.

Calculated for tetrabarium phytate $C_6H_{16}O_{17}P_4Ba_4$ = 1255:

C = 5.73; H = 1.27; P = 14.82; Ba = 43.74; $10 H_2O$ = 12.54 per cent.

The second crystalline compound mentioned above gave the following result on analysis:

C = 6.88; H = 1.50; P = 15.94; Ba = 37.38 per cent.

P = 16.28; Ba = 37.21 per cent.

This corresponds to a tribarium salt.

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A further quantity of the free acid was prepared from this salt by the usual method. From 7 grams of the substance practically the theoretical quantity of acid was obtained. After drying at 100° the substance was analyzed:

0.2319 gram substance gave 0.0767 gram H_2O and 0.0925 gram CO_2 .

After drying over boiling chloroform over phosphorus pentoxide:

0.2378 gram substance gave 0.0703 gram H_2O and 0.0961 gram CO_2 .

0.1495 gram substance gave 0.1414 gram $Mg_2P_2O_7$.

Found: C = 10.87; H = 3.70 per cent.

C = 11.02; H = 3.30; P = 26.36 per cent.

Calculated for phytic acid, $C_8H_{24}O_{27}P_6$ = 714:

C = 10.08; H = 3.36; P = 26.05 per cent.

This preparation gave the same reactions as those previously mentioned. When carefully prepared the acid is a thick colorless syrup readily soluble in water and alcohol. Attempts were made to prepare crystalline salts of the acid with organic bases like pyridine and brucine but without success. These salts could not be obtained in crystalline form. In every case they separated as thick liquids which could not be brought to crystallize even after long standing.

The reaction of the aqueous solution of the acid with inorganic bases may be briefly stated as follows:

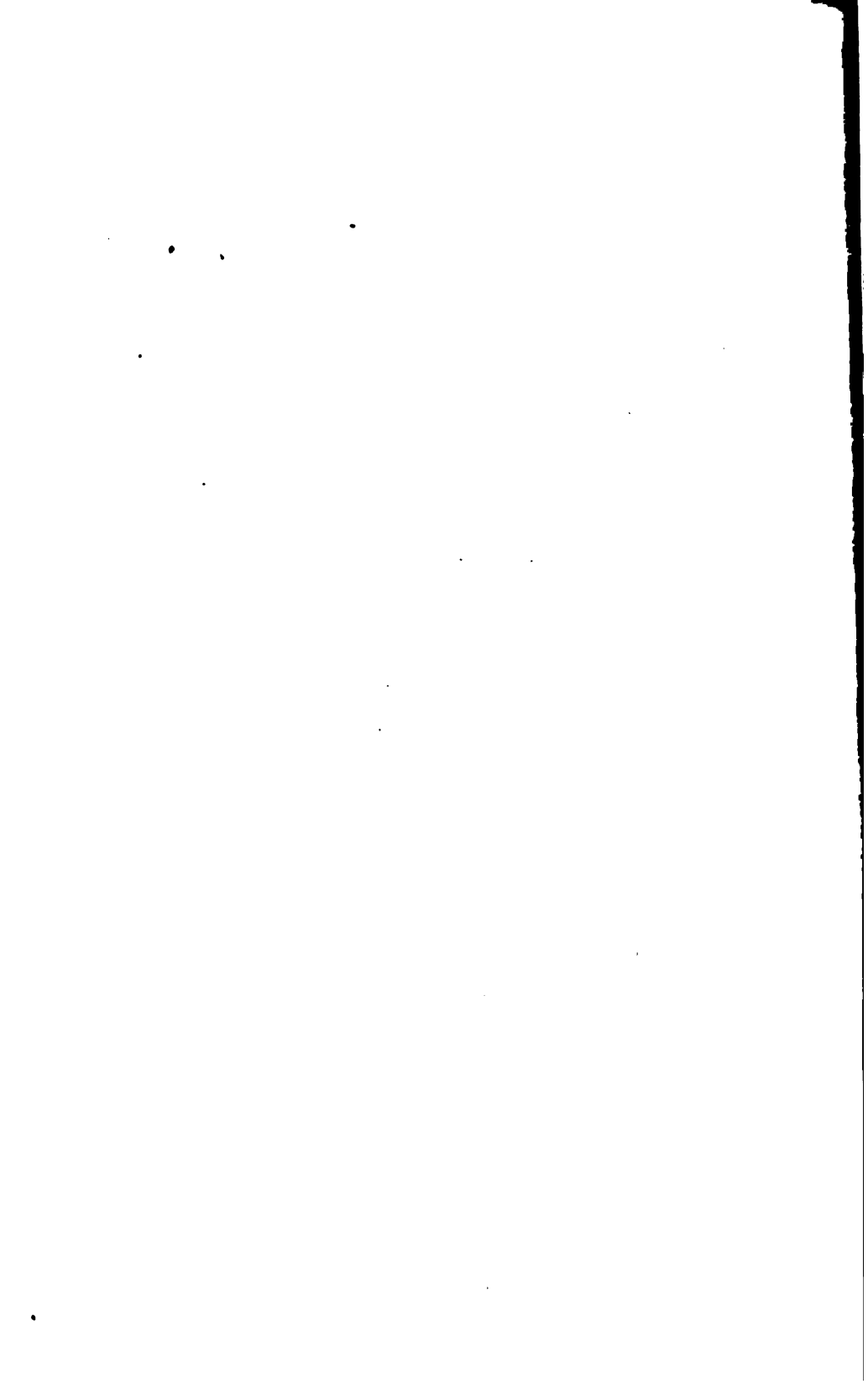
Ammonium molybdate gives a heavy, white, crystalline precipitate. Silver nitrate in excess produces a heavy, white, amorphous precipitate. Magnesia mixture also gives a voluminous, white, amorphous precipitate. It is not precipitated by the chlorides of the alkaline earths but the acetates and hydroxides produce white amorphous precipitates. While barium chloride does not give any precipitate if the solution is allowed to stand at room temperature over night or longer the barium salt crystallizes out in delicate, needle-shaped crystals. In shape and arrangement these crystals are identical with those previously referred to but they are much larger. It immediately coagulates egg albumen. A neutral solution of the acid does not reduce Fehling's solution; even after boiling with dilute hydrochloric acid for some time no reduction takes place. No appreciable color reaction is given with phloroglucine or orcin.

Ferric chloride gives a white precipitate very sparingly soluble in hydrochloric acid. Copper acetate in excess gives a bluish-white precipitate.

On drying at 78° or 100° the substance turns very dark in color but on drying at 60° the color changes but slightly. All the barium salts obtained were strongly acid in reaction on moist litmus paper.

It is evident that the substance isolated from cotton seed meal is very similar to phytin. The various salts which have been analyzed show but little difference in composition as compared with the corresponding phytin derivatives. It may be noted, however, that the analytical results of the purified, so-called tribarium salts point to the empirical formula $C_2H_4P_2O_8Ba$. Such a compound might be a monobasic acid of the formula CH_3PO_4 , but it is also isomeric with inosite hexaphosphate and accordingly differs very little in composition from phytic acid.

If the organic phosphoric acid in cotton seed meal is identical with phytin, if it is an isomer or if it is a somewhat differently constituted substance can hardly be determined from the data presented in this paper. The investigation will be continued.



STUDIES IN THE BLOOD RELATIONSHIP OF ANIMALS AS DISPLAYED IN THE COMPOSITION OF THE SERUM PROTEINS. I.

A COMPARISON OF THE SERA OF THE HORSE, RABBIT, RAT AND OX WITH RESPECT TO THEIR CONTENT OF VARIOUS PRO- TEINS IN THE NORMAL AND IN THE FASTING CONDITION.

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I. METHODS AND RESULTS.

A. *Methods.*

I have shown in a previous communication¹ that it is possible, with a very fair and a thoroughly definite degree of accuracy, to determine the total quantity and relative proportion of each of three groups of serum proteins by a refractometric method, only employing very small quantities (not necessarily exceeding 20 cc.) of serum. The method, briefly recapitulated with slight modifications indicated by further experience, is as follows:

1. An accurately measured volume, usually 10 cc., of fresh whipped and centrifugalized serum is diluted to ten times its volume with distilled water. Carbon dioxide is bubbled through this solution at a good rate (two or three bubbles per second) for at least one hour. The precipitate which is thus obtained is allowed to settle for from twelve to sixteen hours. The supernatant fluid is then carefully decanted and centrifuged in two 50 cc. centrifuge tubes to throw down any flocculi carried over in decantation. Meanwhile, the glass cylinder containing the precipitate is filled up to the 100 cc. mark with distilled water and the contents thoroughly mixed by shaking. A few minutes' energetic centrifugalization of the mother-liquor suffices to throw down any traces of globulin which it contains, and the fluid is then

¹ T. Brailsford Robertson: this *Journal*, xi, p. 179, 1912.

decanted from the centrifuge tubes, leaving the precipitate closely packed at the bottom. These tubes are then filled with the suspension of globulin in distilled water, the last traces of globulin being washed out of the cylinder with a few cubic centimeters of distilled water. These washings are added to the contents of the centrifuge tubes. The suspension is now energetically centrifuged and in a few minutes the precipitate is found to be tightly packed at the bottom of the tubes. The fluid is now poured off, 1 cc. of $\frac{N}{10}$ KOH or NaOH is added to the contents of one of the tubes and 2 or 3 cc. of distilled water. As soon as all of the globulin is dissolved in the first tube, the contents are transferred to the second tube, the first tube being carefully rinsed with 3 or 4 cc. of distilled water. As soon as the globulin in the second tube is completely dissolved, the contents and rinsings from this tube are transferred to a narrow calibrated² measuring cylinder, which is then filled by the addition of distilled water to the 10 cc. mark, that is, to the volume originally occupied by the serum from which the globulin was precipitated.³ The refractive index of this solution and that of $\frac{N}{10}$ KOH or NaOH are determined at the same temperature. The difference between the two readings, divided by 0.00229, yields the *percentage of "insoluble" globulin in the original serum.*

2. To an accurately measured volume of the same serum is added an equal volume of saturated ammonium sulphate solution and the globulins thus precipitated are filtered off; the filtrate is collected and diluted to one-half with water and the refractive index of the mixture thus obtained is measured. At the same time we measure the refractive index of a one-fourth saturated solution of ammonium sulphate prepared by adding to a portion of the saturated ammonium sulphate solution an equal volume of distilled water and diluting the solution thus obtained to one-half. The difference between these two readings, multiplied by 4 and then diminished by 0.00157 (the refractivity of the non-protein constituents of serum) yields the *total refractivity of the albumins of the serum.* If crystallizable albumin be absent,⁴ then this figure divided by 0.00177 yields the *percentage of albumin in the original serum.*

3. The refractive index of the original serum is determined and that of $\frac{M}{11}$ NaCl. The difference between the two readings yields the *total refrac-*

² Calibrated against the pipette employed to measure out the original volume of serum.

³ The centrifugalization of the globulin saves time and also prevents loss in decantation. If, however, the globulin be centrifuged immediately after the passage of the CO₂, it carries down any haemoglobin which the serum may contain and this color cannot be washed out of it again. If, on the contrary, it be allowed to stand for some hours to flocculate it comes down uncolored even from serum tinged with haemoglobin. This fact would appear to indicate danger of contamination by "occlusion," if the precipitate be immediately centrifuged, and the results obtained might conceivably be too high. Hence the procedure indicated above is preferred.

⁴ Cf. below.

tivity of the proteins in the serum. Subtracting the refractivity of the albumins determined above, we obtain the total refractivity of the globulins in the serum. This estimate divided by 0.00229 yields the total percentage of globulins in the serum.

4. The sum of the percentage of albumin and globulin yields the total percentage of proteins in the serum. If desired this estimate may be checked by a Kjeldahl determination of the nitrogen in the total coagulable protein contained in a measured sample of the serum employed.

According to Reiss⁵ the refractivity of crystallized serum albumin differs considerably from that of amorphous serum albumin, 1 gram of the former per 100 cc. of solvent producing a change of 0.00201 in its refractive index while 1 gram of amorphous albumin per 100 cc. of solvent produces a change of 0.00177 in its refractive index. If appreciable quantities of crystallizable serum albumin exist as such in a sample of serum under investigation we should expect to detect its presence by a lack of agreement between the percentage of total proteins, determined refractometrically, and the percentage of total proteins determined by coagulation and a Kjeldahl estimate of the total nitrogen in the coagulum. As we shall see, however, even when serum known to yield large amounts of crystalline albumin to appropriate treatment is employed, to wit, horse serum, no such lack of agreement can be detected. Consequently the method of analysis outlined above yields reliable results whether the serum under examination readily yields crystalline albumin or not.

B. Results obtained with horse serum.

Through the kindness of my colleagues, Drs. C. M. Haring and C. L. Roadhouse, to whom my sincere thanks are due, I have been able to obtain horse serum from an adult animal which had been deprived of food for about twenty-four hours previous to the bleeding.⁶ The blood was collected in Erlenmeyer flasks, directly from the jugular vein, defibrinated by shaking with glass beads and immediately centrifuged. The clear serum was pipetted off and analyzed immediately.

⁵ E. Reiss: *Beitr. z. chem. Physiol. u. Path.*, iv, p. 150, 1903; *Arch. f. exp. Path. u. Pharm.*, li, p. 118, 1903.

⁶ Cf. under sections C and D regarding the influence of fasting upon the composition of the serum-proteins.

The "insoluble" globulin was determined in two 10 cc. samples; the albumins were determined in two 5 cc. samples. In each case the different samples yielded identical results. In these and in all of the other determinations reported in this article a Pulfrich refractometer, reading to within 1' of the angle of total reflection, was employed. A sodium flame was the source of light. The following were the results obtained:

TABLE 1. *Horse serum.*

	per cent.
"Insoluble" globulin.....	0.34 \pm 0.04
Total globulins.....	3.5 \pm 0.15
Total albumins.....	4.6 \pm 0.2
Total proteins.....	8.1 \pm 0.2

The figure following the \pm sign is the possible error in the determination due to a possible error of $\pm 1'$ in reading the angle of total reflection.⁷

Horse serum, as is well known,⁸ readily yields crystalline albumin upon appropriate treatment; accordingly I expected to find a divergence between the percentage of total protein as determined above and the percentage of total proteins estimated by coagulation and the determination of the nitrogen in the coagulum. No such divergence was found, however.

The determination of the total coagulable protein was carried out as follows, three separate 5 cc. samples of the serum being employed:

A 9 cm. S & S 589 "blue band" filter was about three-fourths filled with absolute alcohol and fresh alcohol was kept dripping into the filter from a burette as rapidly as it passed through. A 5 cc. sample of the serum was then delivered very slowly, drop by drop, into the alcohol, which passed through quite clear and devoid of precipitate. The filter and contained coagulum were dried at 36° over H₂SO₄ for forty-eight hours. The nitrogen in the filter and contained precipitate was then determined by the U. S.

⁷ In my previous communication, referred to above, the possible error in determining the total proteins and the albumins is erroneously stated to be ± 0.1 per cent instead of ± 0.2 per cent, while the possible error in determining the total globulin is erroneously stated to be ± 0.1 per cent instead of ± 0.15 per cent.

⁸ Cf. Fr. N. Schulz: *Die Krystallisation von Eiweissstoffen*, Jena, 1901, p. 13.

official Kjeldahl method.⁹ The nitrogen was multiplied by 6.29 in estimating the protein. The following were the results obtained:

TABLE 2. *Alcohol-coagulable protein in horse serum.*

SAMPLE	N IN SAMPLE	PER CENT OF ALCOHOL- COAGULABLE PROTEIN IN THE SERUM
	<i>mgm.</i>	
1	64.8	8.15
2	62.8	7.90
3	63.3	7.97
Average: 8.00		

Hence, within the experimental error of the determinations, the percentage of alcohol-coagulable protein in horse serum is identical with the percentage of total proteins determined on the assumption that all of the albumins in the serum have a refractivity per gram per 100 cc. of solvent of 0.00177. It appears, therefore, that albumin of a refractivity of 0.00201 per gram per 100 cc. of solvent is not present in sufficient amount in horse serum to appreciably affect the accuracy of refractometric determinations carried out as outlined above. The reason for this is readily perceived when we consider the magnitude of the divergence between the above estimates which we should expect to find were the *whole* of the serum albumin of the crystallizable variety. In this event, it appears, from the refractometric observations, that in the serum under examination the percentage of total albumin would be 4.1 and the percentage of total proteins, 7.6. No less than 40 per cent of the total albumins would have to be of the crystallizable variety, therefore, in order to cause a divergence of 0.2 per cent between the two estimates of the total proteins. Now, as we have seen, the possible error in the refractometric determination of the percentage of total proteins is \pm 0.2 per cent. Hence we may draw the following conclusions:

- (1) Horse serum does not contain preformed crystallizable albumin to the extent of over 40 per cent of the total albumins.
- (2) The refractometric method cannot be employed to determine more precisely than this the quantity of crystallizable albumin contained in sera.

⁹ U. S. Department of Agric., Bureau of Chemistry, Bulletin 107, p. 5, 1910.

(3) Provided not over 40 per cent of the albumins are of the crystallizable variety no appreciable error is introduced into the refractometric determinations of the total albumins and total proteins in sera by assuming that all of the albumins present have a refractivity of 0.00177 per gram per 100 cc. of solvent.

Expressing the quantity of each of the proteins in the horse serum employed in terms of the percentage of the total proteins which they represent, the above results may be summarized as follows:

"Insoluble" globulin.....	4.2	(\pm 0.4)
Total globulins.....	43	(\pm 2)
Total albumins.....	57	(\pm 2)

The figures in brackets represent the plus or minus error in the estimation of these percentages which would be brought about by an error of 1' in reading the angle of total reflection.

These results are not in good agreement with those obtained by Hammarsten¹⁰ and by Lewinski,¹¹ who agreed in stating that the percentage of globulins exceeds the percentage of albumins in the serum of the horse. I have had occasion to note a similar divergence between my results and those of Hammarsten, employing ox serum.¹² This divergence is the more surprising since, as we shall see, the agreement between my results and those of Hammarsten and other observers employing the serum of fully fed (*i.e.*, "normal") rabbits is highly satisfactory. In view of the marked influence of fasting upon the relative proportion of the globulins and albumins in blood sera, which we will discuss in sections C and D of this article, I am inclined to attribute these divergencies to the fact that the horse and oxen from which I obtained my sera were in a fasting condition,¹³ while those employed by Hammarsten and Lewinski were not improbably fully fed animals. I am confirmed in this belief by the fact that in one instance Hammarsten obtained ox serum containing a greater percentage of albumins than of globulins.

¹⁰ O. Hammarsten: *Arch. f. d. ges. Physiol.*, xvii, p. 413, 1878.

¹¹ J. Lewinski: *ibid.*, c, p. 611, 1903.

¹² T. Brailsford Robertson: *loc. cit.*

¹³ The oxen killed at local slaughter-houses are invariably fasting and have usually travelled long distances by railway without food.

C. Results obtained with rabbit serum.

I have carried out a number of analyses of the blood serum of rabbits. The animals employed were all of medium size (2000 grams or thereabouts) and were bled directly from the carotid artery into a small Erlenmeyer flask containing glass beads, which were agitated until the blood was defibrinated. The blood was then immediately centrifugalized, and the clear serum pipetted off and analyzed.

For my earlier analyses I employed serum obtained from "normal" animals, animals, that is, taken directly from the animal house and fed at an undetermined time prior to the bleeding. The following were the results obtained.

TABLE 3. *Normal rabbits.*

RABBIT NUMBER	"INSOLUBLE" GLOBULINS	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1 (Gray).....		1.7 \pm 0.15	4.9 \pm 0.2	6.6 \pm 0.2
2 (Yellow).....	0.35 \pm 0.04	2.0 \pm 0.15	4.5 \pm 0.2	6.5 \pm 0.2
3 (Yellow).....		2.1 \pm 0.15	5.0 \pm 0.2	7.1 \pm 0.2
Average.....	0.35 \pm 0.04	1.9 \pm 0.15	4.8 \pm 0.2	6.7 \pm 0.2

Expressing each of the above mentioned proteins or groups of proteins in terms of the percentage of the total proteins which they represent, the above results for "normal" rabbit serum may be summarized as follows:

"Insoluble" globulin.....	5.2	(\pm 0.4)
Total globulins.....	28	$\left\{ \begin{smallmatrix} 31 \\ 26 \end{smallmatrix} \right\}$ (\pm 2)
Total albumins.....	72	$\left\{ \begin{smallmatrix} 74 \\ 69 \end{smallmatrix} \right\}$ (\pm 2)

The first figure opposite each group represents the average percentage; the upper figures immediately following, the highest percentage observed in any individual; the lower figure, the lowest percentage observed in any individual; and the figure in brackets, the plus or minus error in the estimation of these percentages which would be brought about by an error of 1' in reading the angle of total reflection.

From these results, it would appear that the variations in the relative proportions of the three groups of proteins considered

in the three different individuals employed are not greater than the variations which might arise from experimental errors in the determinations. The above results may be compared with the following previous estimates of the total globulins, albumins and total proteins in rabbit serum.

O. Hammarsten;¹⁴ determinations made upon four animals; globulins precipitated by saturation of the serum with $MgSO_4$:

Total globulins.....	29	{ 32 25
Total albumins.....	71	{ 75 68

C. Inagaki;¹⁵ determinations made upon thirteen animals; globulins precipitated by half-saturation of the serum with Am_2SO_4 :

Total globulins.....	29	{ 36 21
Total albumins.....	71	{ 79 64

These data, obtained by precipitation of the globulins and subsequent Kjeldahl determinations of the nitrogen contained in the precipitate and in the coagulated proteins of whole serum are in good accord with the data obtained by the much less laborious refractometric method. The individual variations in the ratio of globulin to albumin which are recorded by Hammarsten and Inagaki are, however, large—much too large it would appear to be attributed to experimental errors in the determinations. It occurred to me, however, that these variations might in large part be attributable to the presence in the blood of varying quantities of protein absorbed from the intestinal tract, and the possibility suggested itself that the relative proportions of the serum proteins in a fasting animal might be less subject to individual variation and more typical of the animal species under investigation. Accordingly, a number of determinations, similar to the above, were carried out, the rabbits employed, however, being animals which had fasted, with free access to water, for a period of five days preceding bleeding. The following were the results obtained:

¹⁴ O. Hammarsten: *Arch. f. d. ges. Physiol.*, xvii, p. 459, 1878.

¹⁵ C. Inagaki: *Zeitschr. f. Biol.*, xlix, p. 77, 1907.

TABLE 4. *Fasting rabbits.*

RABBIT NUMBER	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1 (Gray).....		1.2 \pm 0.15	6.8 \pm 0.2	8.0 \pm 0.2
2 (Gray).....		1.4 \pm 0.15	7.2 \pm 0.2	8.6 \pm 0.2
3 (Gray).....	0.21 \pm 0.04	1.3 \pm 0.15	6.2 \pm 0.2	7.5 \pm 0.2
4 (Black).....	0.21 \pm 0.04	1.2 \pm 0.15	6.8 \pm 0.2	8.0 \pm 0.2
5 (Gray).....	0.24 \pm 0.04	1.3 \pm 0.15	5.6 \pm 0.2	6.9 \pm 0.2
6 (Gray).....	0.24 \pm 0.04	1.2 \pm 0.15	6.2 \pm 0.2	7.4 \pm 0.2
7 (long haired albino)	0.24 \pm 0.04	1.3 \pm 0.15	6.0 \pm 0.2	7.3 \pm 0.2
Average.....	0.23 \pm 0.04	1.3 \pm 0.15	6.4 \pm 0.2	7.7 \pm 0.2

Expressing each of the above mentioned proteins or groups of proteins in terms of the percentage of the total proteins which they represent, these results for fasting rabbit's serum may be summarized as follows:

"Insoluble" globulins..... 3.0 $\left\{ \begin{smallmatrix} 3.5 \\ 2.6 \end{smallmatrix} \right\}$ (\pm 0.4)

Total globulins..... 17 $\left\{ \begin{smallmatrix} 18 \\ 15 \end{smallmatrix} \right\}$ (\pm 2)

Total albumins..... 83 $\left\{ \begin{smallmatrix} 85 \\ 82 \end{smallmatrix} \right\}$ (\pm 2)

From these results we perceive:

(1) That the percentage of total proteins in sera derived from different fasting individuals is highly variable, a fact which is readily understood when we reflect that the animals were allowed free access to water.

(2) That the relative proportions of the different proteins in the sera of fasting animals are constant within the limits of the experimental error of the determination.

(3) That during starvation the total protein content of the blood serum increases (compare tables 3 and 4).

(4) That during starvation the percentage of globulins in the blood serum diminishes.

(5) That, consequently, during starvation the proportion of albumins to globulins undergoes a marked increase.

An increase in the total protein content of blood serum during starvation has been observed by Simon¹⁶ in the horse and by Lewinski¹⁷ and Githens¹⁸ in the dog. The period of starvation employed by Simon and Lewinski being from three to six days, while that employed by Githens was from two to three weeks. Salvioli¹⁹ and Burckhardt²⁰ employing a period of from three to six days' starvation in dogs found no perceptible alteration in the protein content of the serum, while Panum²¹ employing a period of thirteen days' starvation in dogs found an actual decrease in the total protein content of the serum.

Having regard to the fact that the methods of determining the protein content of fluids have undergone great improvement since Panum, Salvioli and Burckhardt carried out their estimations, these results are in good accord with my own results cited above. As regards the alteration in the proportion of globulins to albumins consequent upon starvation, however, much divergence of opinion is expressed by different investigators. Thus Salvioli finds no alteration in the proportion of globulins to albumins in the blood serum of dogs during starvation, while Burckhardt, also employing dogs, finds an increase in the "insoluble" globulin (precipitated by dialysis) and a corresponding decrease in the percentage of residual protein. Lewinski, who separates the globulins by saturation with $MgSO_4$, finds an increase both in globulins and in albumins in dog serum during starvation, the increase in globulins being more marked than the increase in albumins. Githens, who separates the globulins by fractional coagulation with $NaCl$, finds a decrease in the proportion of albumins and a corresponding increase in the proportion of globulins in dog serum during starvation. The consensus of opinion appears to be, therefore, that in dogs fasting leads to an increase in the proportion of globulins to albumins.

¹⁶ Simon: *Physiologische und pathologische Anthropechemie*, Berlin, 1842, p. 235, cited after Panum: *Virchow's Archiv*, xxix, p. 241, 1864.

¹⁷ J. Lewinski: *Arch. f. d. ges. Physiol.*, c, p. 611, 1903.

¹⁸ T. Githens: *Beitr. z. chem. Physiol.*, v, p. 514, 1904; cf. also V. Subbotin: *Zeitschr. f. Biol.*, vii, p. 185, 1871; J. G. Otto: *Maly's Jahresbericht f. Tierchem.*, xvii, p. 134, 1888.

¹⁹ G. Salvioli: *Arch. f. (Anat. u.) Physiol.*, 1881, p. 269.

²⁰ Burckhardt: *Arch. f. exp. Path. u. Pharm.*, xvi, p. 322, 1883.

²¹ P. D. Panum: *loc. cit.*

H. T. Krieger,²² however, employing rabbits, obtains the same result as I, namely, a *decrease* in the proportion of globulins to albumins during starvation. It appears, therefore, that the direction in which this ratio alters during starvation varies with the species, possibly with the nature of the normal dietary of the animal. As we shall see, my results obtained with the sera of normal and of fasting rats confirm this view.

D. Results obtained with rat serum.

Twelve normal (that is, fully fed) adult albino rats were etherized and bled by cutting the throat. The blood of all of the animals was caught in the same Erlenmeyer flask containing glass beads and defibrinated by shaking. It was then immediately centrifuged, and the clear serum pipetted off and analyzed. The following were the results obtained:

TABLE 5. *Normal rats.*

	<i>per cent.</i>
"Insoluble" globulin.....	0.48 \pm 0.04
Total globulin.....	1.7 \pm 0.15
Total albumins.....	4.8 \pm 0.2
Total proteins.....	6.5 \pm 0.2

Expressing the above results, as in previous cases, in terms of percentages of the total proteins, we obtain for normal rat serum:

"Insoluble" globulin.....	7.4 (\pm 0.4)
Total globulin.....	26 (\pm 2)
Total albumins.....	74 (\pm 2)

Similar determinations were carried out upon the serum of fasting rats. Eighty animals were fasted for thirty-six hours.²³ They were bled in batches of forty into two separate Erlenmeyers, the blood being defibrinated with glass beads and then centrifuged as before. The following were the results obtained:

²² H. T. Krieger: *Inaugural Dissertations Strassburg*, 1899, cited after *Maly's Jahresbericht f. Tierchem.*, 1899, p. 14.

²³ It is impossible to fast rats much longer than this without inducing pathological symptoms. After sixty hours they die of hunger or else, if kept together, commence devouring each other.

TABLE 6. *Fasting rats.*

	BATCH 1. per cent.	BATCH 2. per cent.
"Insoluble" globulin.....	0.55 \pm 0.04	0.55 \pm 0.04
Total globulins.....	2.6 \pm 0.15	2.7 \pm 0.15
Total albumins.....	4.8 \pm 0.2	4.8 \pm 0.2
Total proteins.....	7.4 \pm 0.2	7.5 \pm 0.2

Whence we may conclude:

(1) That in rats, as in rabbits and dogs, starvation leads to an increase in the protein content of the blood serum.

(2) That in rats, as in dogs, the effect of starvation upon the ratio of globulin to albumin is contrary to its effect upon this ratio in rabbits, that is to say, the proportion of globulins to albumins *increases*.

Expressing the above results, as in previous cases, in terms of percentages of the total proteins, we obtain for fasting rat's serum:

"Insoluble" globulin.....	7.4 (\pm 0.4)
Total globulins.....	36 (\pm 2)
Total albumins.....	64 (\pm 2)

II. DISCUSSION OF THE RESULTS.

Summarizing the results enumerated above, obtained with fasting animals, and comparing them with the results previously obtained with the serum of fasting oxen, we obtain the following table in which the results are expressed as percentages of total protein:

TABLE 7. *Fasting animals.*

SPECIES	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS
Albino rat.....	7.4 (\pm 0.4)	36 (\pm 2)	64 (\pm 2)
Rabbit.....	3.0 $\left\{ \begin{array}{l} 3.5 \\ 2.6 \end{array} \right\}$ (\pm 0.4)	17 $\left\{ \begin{array}{l} 18 \\ 15 \end{array} \right\}$ (\pm 2)	83 $\left\{ \begin{array}{l} 85 \\ 82 \end{array} \right\}$ (\pm 2)
Ox.....	8.9 (\pm 0.4)	36 (\pm 2)	64 (\pm 2)
Horse.....	4.2 (\pm 0.4)	43 (\pm 2)	57 (\pm 2)

Assuming that the divergence between my results for horse and ox sera and those of previous observers, alluded to above, is due to the fact that I employed the sera of fasting animals, while others have employed the sera of fully fed animals, we may construct

a similar tabular comparison of the sera of "normal," *i.e.*, fully fed animals, as follows:

TABLE 8. *Normal animals.*

SPECIES	"INSOLUBLE" GLOBULINS	TOTAL GLOBULINS	TOTAL ALBUMINS
Albino rat (Robertson).....	7.4 (± 0.4)	26 (± 2)	74 (± 2)
Rabbit (Robertson).....	5.2 (± 0.4)	28 $\left\{ \begin{smallmatrix} 31 \\ 26 \end{smallmatrix} \right\}$ (± 2)	72 $\left\{ \begin{smallmatrix} 74 \\ 69 \end{smallmatrix} \right\}$ (± 2)
Rabbit (Hammarsten).....		29 $\left\{ \begin{smallmatrix} 32 \\ 25 \end{smallmatrix} \right\}$	71 $\left\{ \begin{smallmatrix} 75 \\ 68 \end{smallmatrix} \right\}$
Rabbit (Inagaki).....		29 $\left\{ \begin{smallmatrix} 36 \\ 21 \end{smallmatrix} \right\}$	71 $\left\{ \begin{smallmatrix} 79 \\ 64 \end{smallmatrix} \right\}$
Ox (Hammarsten*).....		58 $\left\{ \begin{smallmatrix} 64 \\ 54 \end{smallmatrix} \right\}$	42 $\left\{ \begin{smallmatrix} 46 \\ 36 \end{smallmatrix} \right\}$
Horse (Hammarsten†).....		63 $\left\{ \begin{smallmatrix} 71 \\ 58 \end{smallmatrix} \right\}$	37 $\left\{ \begin{smallmatrix} 42 \\ 29 \end{smallmatrix} \right\}$
Horse (Lewinski‡).....		63 $\left\{ \begin{smallmatrix} 69 \\ 56 \end{smallmatrix} \right\}$	37 $\left\{ \begin{smallmatrix} 44 \\ 31 \end{smallmatrix} \right\}$

* Determinations made upon four individuals: the fifth, alluded to above (Section B), in which the albumins were present in excess of the globulins is omitted.

† Determinations made upon ten individuals.

‡ Determinations made upon four individuals.

A comparison of these tables enables us to draw the following inferences:

(1) In the rabbit, ox and horse, which are herbivorous animals, starvation leads to an increase in the proportion of albumins to globulins in serum, while in the rat, which is omnivorous, and (*cf.* above) in the dog, which is carnivorous, starvation leads to an increase in the proportion of globulins to albumins in the serum. These facts, although as yet insufficiently extended to afford a safe basis for generalization, suggest rather forcibly the view that the effect of starvation upon the ratio of globulin to albumin in mammalian blood sera is correlated with the nature of the normal dietary and, conversely, that the nature of the normal dietary is determined by the composition of the blood and the needs of the tissues no less than by the character of the alimentary canal. If this be indeed the case then the effect of starvation upon the rats employed in my investigations is very surprising in view of the fact that they had been fed for many weeks preceding the analyses upon a *purely vegetable diet*, to wit, crushed barley.

The great constancy of the ratio of "insoluble" globulin to total globulin and to total albumins, in different fasting individuals of the same species (cf. table 4), and its variation in the different species investigated, indicate that it is a specific character. In fully fed animals the individual variations are greater; nevertheless the data enumerated in table 8 indicate that the average value of this ratio is a specific character for fully fed animals also. A comparison of tables 7 and 8 would even appear to indicate that phylogenetic relationships are better displayed in the protein composition of the sera of "normal" than in that of the sera of fasting animals, for in table 8 the two rodent sera are clearly seen to be related and differ markedly from the two ungulate sera which also resemble one another. In table 7 these relationships are not so clearly displayed. As yet, the data at hand are too scanty to enable one to determine in favor of the one or of the other standard of comparison. A comparison between the sera of fully fed animals, however, obviously necessitates a much larger number of determinations than a comparison between the sera of fasting animals.

I have elsewhere²⁴ pointed out that we are in possession of many data tending to show that the proteins in blood serum are united to form a complex, the character of which is presumably determined by the relative proportions in which the different proteins enter into it, and I have suggested that the specific character of this complex in different species may possibly underlie the phenomenon of specificity in the immunological sense. The fact that starvation alters the proportion in which the various proteins are present in the sera of animals does not conflict with this view, while the high degree of constancy in the relative proportion of the various proteins in the sera of fasting animals lends some degree of support to it.

The investigations of Voit and the more modern investigations of Folin,²⁵ Schreuer²⁶ and others have taught us to distinguish between *circulating protein*, which is only transitorily resident in the body, and *tissue protein*, which is bound up with the maintenance of life and not readily catabolized. We may well suppose

²⁴ T. Brailsford Robertson: *Univ. of Calif. Publ. Physiol.*, iv, p. 25, 1911; *Die physikalische Chemie der Proteine*, Dresden, 1912, pp. 126-132.

²⁵ O. Folin: *Amer. Journ. of Physiol.*, xiii, p. 117, 1904.

²⁶ M. Schreuer: *Arch. f. d. ges. Physiol.*, cx, p. 227, 1905.

that protein of the former character, derived from the alimentary canal and in process of being carried to the tissues by the blood stream, does not form an integral part of the specific serum-protein complex to which I have referred. In fully fed animals such "circulating protein" would be present in the blood in *maximum* concentration, while in the blood of fasting animals, in accordance with the metabolic "law of minimum," we should expect "circulating" readily catabolized protein to be present in *minimum* concentration.

III. SUMMARY.

The quantities of "insoluble" globulin, total globulin and total albumin contained in the sera of the normal (fully fed) rat and rabbit and of the fasting horse, rat and rabbit have been determined by a refractometric method described in a previous communication. During the course of this investigation the following conclusions were reached:

(1) The refractometric method of analysis yields results (with the rabbit) which closely agree with those obtained by previous observers, employing other methods of analysis.

(2) Horse serum does not contain preformed crystallizable albumin to the extent of more than 40 per cent of the total albumins.

(3) The refractometric method cannot be employed to determine more precisely than this the quantity of crystallizable albumins contained in sera.

(4) Provided not over 40 per cent of the albumins are of the crystallizable variety, no appreciable error is introduced into the refractometric determinations of the total albumins and total proteins in sera by assuming that all of the albumins present have the refractivity of amorphous serum albumin, to wit, 0.00177 per gram per 100 cc. of solvent.

(5) The relative proportion in which the above mentioned three groups of proteins are present in the serum of fully fed animals is subject to rather high individual variations; the *average* values are, however, characteristic of the species from which the serum is derived.

(6) The percentage of total proteins in sera derived from different fasting individuals (with free access to water) is highly variable.

(7) The relative proportions in which the above mentioned three groups of proteins are present in the sera of fasting animals of the same species (rabbits) is constant within the limits of the experimental error of the determination.

(8) During starvation, the total protein content of the blood-serum rises.

(9) In the rabbit, ox and horse starvation leads to an increase in the proportion of albumins to globulins in the serum, while in the rat and in the dog starvation leads to an increase in the proportion of globulins to albumins in the serum.

PUTREFACTION WITH SPECIAL REFERENCE TO THE PROTEUS GROUP.

BY LEO F. RETTGER AND CLYDE R. NEWELL.

(From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University.)

(Received for publication, October 15, 1912.)

The subject of putrefaction has caused much confusion in the minds of different investigators. Most of the definitions of the term putrefaction which may be found in text-books, dictionaries, etc., are so vague and in many instances contradictory that little information may be gained from them. Some authors regard all bacterial decompositions of organic matter, particularly proteins, carbohydrates and fats, as putrefactive changes. Others would limit the action to proteins alone without special reference to the actual nature of the decomposition or to the products. Others, again, would restrict the meaning so as to include only those processes of protein disintegration which give rise to foul-smelling products.

In previous publications¹ one of us accepted the last-mentioned view, namely, that of Bienstock² and numerous other investigators. Since then there has been no occasion to depart from that view. It may be said that there is no word in the English language which conveys exactly the meaning that the word "Fäulniss" does; yet the term "putrefaction" is of practically the same significance. It is derived from the Latin "putrere," meaning "to be rotten," and hence may be defined literally as a process of rotting or offensive decay.

There is no very serious objection to giving the word putrefaction the more popular meaning which is so generally applied to it, as a matter of convenience, but in its scientific application its meaning should be more specific or restricted. Putrefaction

¹ Rettger: this *Journal*, ii, p. 71, 1906; *ibid.*, iv, p. 45, 1908.

² Bienstock: *Arch. f. Hyg.*, xxxvi, p. 335, 1899; *ibid.*, xxxix, p. 390, 1901.

may be defined, therefore, as a particular process of protein decomposition which is brought about through the agency of bacteria with the evolution of foul-smelling products which are characteristic of ordinary cadaveric decomposition. It should be stated that mercaptan is of particular significance and that indole, skatole and hydrogen sulphide are of less importance.

Pasteur was the first to point out that putrefaction is essentially an anaërobic process. This view has since been held by many investigators. Certain writers have in recent years attempted to overthrow the conception of Pasteur, however. Whether the newer contentions are based on sufficient experimental evidence may be regarded by many as an open question.

In a previous paper³ it was claimed that only certain obligate anaërobic are able to initiate and carry on the decomposition of native proteins in the absence of atmospheric oxygen. *Bacillus putrificus*, *B. edematis maligni* and *B. anthracis symptomatici* were mentioned as the best examples. *B. tetani* does not have a place in this group. *B. aërogenes capsulatus* (Welch) has only a limited if, in fact, any proteolytic action. All of the facultative anaërobic that were tried were found to lack this power. At least thirty different organisms, including *Proteus vulgaris*, were subjected to the test. The results were in perfect harmony with those which Bienstock⁴ obtained in his extensive work on putrefaction.

During the past two years we have repeated some of the earlier experiments, and particularly those bearing on the proteus group. As a preliminary part of the investigation, we sent personal requests to at least twenty-five different bacteriological laboratories for culture tubes of members of the proteus group of bacilli. We were liberally supplied with cultures from many sources, including four strains from the Pasteur Institute of Paris (Professor Metchnikoff's laboratory). Of the tubes received, twelve were labeled *Proteus vulgaris*, three, *P. mirabilis*, two, *P. zenkeri*, one, *P. hominis* and one, *P. versicolor*. Furthermore, there were tubes bearing the numbers 370, 372, 374 (1) and 374 (2) and four cultures from the Pasteur Institute labeled *Proteus* a, b, c and d.

³ Rettger: this *Journal*, ii, p. 71, 1906.

⁴ Bienstock: *loc. cit.*

All of the strains were put through the most important cultural and microscopic tests in order to satisfy ourselves that we were in each case working with a member of the so-called proteus group and with uncontaminated cultures. The putrefaction tests were usually made in the egg-meat medium¹ which has for several years been employed in this laboratory. Tubes of blood fibrin suspended in bouillon and in Uschinsky medium were employed also. The egg-meat medium was used in preference to others because of the ease with which it is prepared and the satisfactory results which have been obtained with it.

All of the tests were made in large test tubes. Anaërobiosis was induced by means of the alkali-pyrogalllic acid mixture, by the Buchner and the Wright methods. As checks, putrefaction tests were made with *B. putrificus*, *B. edematis maligni* and *B. anthracis symptomatici*. Positive putrefaction results were obtained with these organisms in every instance; that is, on opening the tubes the characteristic odor of putrefaction was given off and the solid matter (egg white and meat) was rapidly reduced in volume.

With the various members of the proteus group the results were entirely different. Not one of the twenty-six strains which were used in this investigation gave us the slightest indication that it possessed the ability to initiate and bring about any visible change in the character of the protein material, when grown under anaërobic conditions. There was no visible reduction in the volume of the solid matter nor the least odor of putrefaction when perfectly clean rubber stoppers were employed in sealing the tubes. In a very few instances a slight odor of putrefaction was perceived, but this was found to be due to stoppers which had been used in positive putrefaction tests. On removing the stopper and cotton plug, and after thorough cleansing of the mouth of the tube, the odor disappeared.

Tubes were left in the incubator (37.5°C.) for at least a week; often for two or three weeks. They were also kept at ordinary room temperature for similar periods. Before incubation at the higher temperature the tubes were kept comparatively cool (8-10°) for from twenty-four to thirty-six hours in order to

¹ Rettger: this *Journal*, ii, p. 71, 1906.

allow the oxygen to be absorbed before any aërobic bacterial action could take place. All of the tests were duplicated and duplicate examinations of each organism were made at least three or four times.

Rigid microscopic examinations were always made of the contents of the proteus-inoculated tubes and, whenever there was any question of contamination, cultural tests were made. The organisms used in the inoculation were always detected with ease and their numbers indicated that considerable multiplication had taken place. The growths were made possible by the presence of non-protein nitrogenous substances and not by the proteins.

Besides the anaërobic tests numerous examinations were made under aërobic conditions. In so far as actual putrefaction is concerned, the results were again all negative. *Proteus zenkeri* has no visible disintegrating action whatever. *P. mirabilis* likewise has none or, at the most, very little when grown under aërobic conditions. On the other hand, *P. vulgaris* possesses the ability to attack and to a certain extent decompose the egg-meat proteins and blood fibrin. Such action is, however, very slow and is dependent on an ample supply of atmospheric oxygen. In such decomposition of protein there are a number of well-known bacterial decomposition products as indole, amines, amino-acids, hydrogen sulphide, etc., but these are not the products of real putrefaction, in the sense in which we use the term, and the odor given off from the *P. vulgaris* tubes is not that which is so characteristic of putrefactive changes, as illustrated in the action of *B. putrificus*.

Many of the common aërobes and facultative anaërobes are known to have definite proteolytic action on egg albumin, serum albumin, etc., when in contact with free oxygen. This is demonstrated easily on ordinary blood serum or egg white when they have been coagulated by heat, and we need not seek any further for organisms than *B. subtilis*, *B. prodigiosus*, *B. pyocyaneus*, etc. Furthermore, indole and hydrogen sulphide are very common protein decomposition products, and are not indicators of real putrefaction. The results are quite different, however, when oxygen is excluded, as we have so frequently observed.

We are forced to disagree, therefore, with Tissier and Martelly,⁶ who claim that *Proteus vulgaris* exerts a putrefactive influence on proteins. When inoculated by them into Uschinsky-Fraenkel medium containing blood fibrin the organism caused the medium to become clouded in twenty-four hours and, according to them, a fetid odor was given off in the course of two days. The fibrin was attacked. At the end of fifteen days the action seemed to be arrested. On analysis they found indole, phenol, amines, leucine, acetic, formic, butyric and valerianic acids and hydrogen sulphide. No mention is made of mercaptan or the aromatic oxy-acids. In its action on proteoses the organism produced the same products.

They succeeded in isolating an enzyme which acts in neutral, alkaline or slightly acid solution, but which is much less active than that of the anaërobes studied. They found *P. zenkeri* associated with *P. vulgaris* in spontaneous putrefaction mixtures, but were unable to demonstrate the presence of a proteolytic enzyme in cultures of *P. zenkeri*. This organism was unable to attack native proteins.

We have no comments to make on the question as to whether the action of *P. vulgaris* on the blood fibrin, as observed by Tissier and Martelly, was one of real putrefaction, in the sense that we use the term. Their observations were made on a very limited number of strains of *P. vulgaris*, if indeed more than one. On the other hand, at least eighteen different strains of the organism were tested by us, and in every instance with negative results.

Basing his conclusions largely on the observations of Tissier and Martelly, Metchnikoff⁷ holds to the view that putrefaction may be induced by certain aërobes.

The putrefactive anaërobes and certain members of the proteus group are frequently associated with each other. While they appear to be present but rarely in the normal intestines of man, they are found side by side in the spontaneous decomposition of protein-containing substances, like meat, humus, etc. The two groups assume different rôles in the processes of disintegration of protein and in putrefaction. Together with other organisms which are essentially aërobic, the *P. vulgaris* exerts an early dis-

⁶ Tissier and Martelly: *Ann. de l'Inst. Pasteur*, xvi, p. 865, 1902.

⁷ Metchnikoff: *ibid.*, xxii, p. 928, 1908.

Putrefaction and Proteus Group

ation action, during the process of which free oxygen is removed from the medium, whereby the putrefying anaërobes are enabled to carry on the process of real putrefaction. The finding of *P. vulgaris* in putrefaction mixtures is not a necessary indication that it has played a part in the putrefaction, as has been assumed by many ever since the discovery of the organism by Trauer. According to Tissier,⁸ *B. proteus vulgaris* rapidly destroys the intermediate cleavage products of proteins. In this way it is undoubtedly an important aid in the complete disintegration of proteins.

Putrefaction is the work of certain obligate anaërobes. If such a function is assumed by any other organisms, as Tissier and Martelly claim, it is rare and of little significance.

⁸ Tissier: *Ann. de l'Inst. Pasteur*, xxvi, p. 522, 1912.

THE BEHAVIOR OF SOME HYDANTOIN DERIVATIVES IN METABOLISM. I.

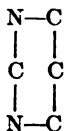
HYDANTOIN AND ETHYL HYDANTOATE.

By HOWARD B. LEWIS.

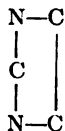
(From the Sheffield Laboratory of Physiological Chemistry, Yale University,
New Haven, Connecticut.)

(Received for publication, October 16, 1912.)

The demonstration of the occurrence of pyrimidine derivatives as constituents of the nucleic acid molecule has awakened a wide interest in the physiological behavior of the pyrimidine ring. The possible biochemical significance of this group is attested by its structural relationship to the purines, creatinine, allantoin and other physiologically important compounds. Although hydantoin and its derivatives have not yet been found present as constituents of any tissues of the body, the behavior of the hydantoin nucleus, a structure similar to the pyrimidine grouping but containing one less carbon atom, deserves consideration in connection with intermediary metabolism.

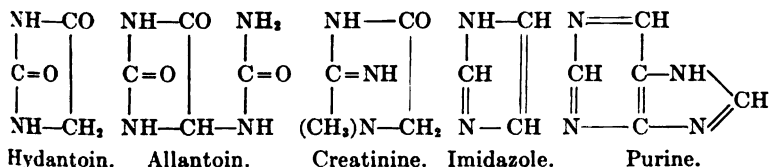


Pyrimidine nucleus.



Hydantoin nucleus.

The close relationship between hydantoin, allantoin, creatinine, purine and imidazole may be seen by a comparison of their structural formulae.



Hydrocarbon Derivatives

...stances with the possible exception of the ...
...has been demonstrated to be destroyed when ...
...Bakin and Wakeman¹ have shown ...
...the liver that some slight decom- ...
...the imidazole nucleus, may ...
...acetoacetic acid, but they con- ...
...to formulate any promising hypoth- ...
...Feeding experiments with ...
...this substance in the organism in ...
...these tested compounds to experience ...
...the behavior of hydantoin, ...
...of particular interest. ...
...the behavior of hydantoin seems ...
...with alloxan and alloxantin

NH—

... ~~... adding~~ $\psi = 0$ functions to stim-

VIII—

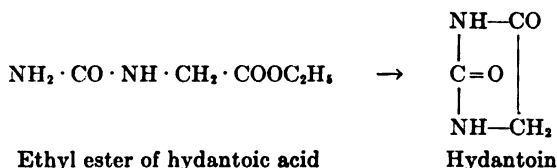
... according to Lusini, ... the stimulating property ... increases the toxicity. More ... Lusini's conclusions since ... and which is non-toxic, ... from the toxic sub- ... contains the alleged

... is of interest.

... of hydantoin and

... lxxii, pp. 322-32.
... 395-99, 1910; Kow-
... 341-57, xxii, pp.
... 1895.
... xi, 22, 44, 75, 193.

the ethyl ester of hydantoic acid, introduced in different ways into the organism of various species. The hydantoic acid ester was prepared from glycocholate hydrochloride and potassium cyanate, according to the method of Harries and Weiss.⁵ On evaporating the ester to dryness with concentrated hydrochloric acid, it is converted to the hydantoin. The latter is then purified by recrystallization from absolute alcohol.



Analysis (Kjeldahl nitrogen determination) of the hydantoin prepared gave the following result:

	Calculated for $\text{C}_3\text{H}_4\text{N}_2\text{O}_2$:	Found:
N.....	28.00 per cent.	27.88 per cent.

For the identification of the hydantoin in the urine, use was made of the insoluble benzalhydantoin. The urine was acidified and evaporated to small volume on a water bath, decolorized with animal charcoal and evaporated to dryness. The product was then condensed with benzaldehyde in the presence of glacial acetic acid, acetic anhydride and dried sodium acetate, as described by Wheeler and Hoffman.⁶ For the identification of the ester, the urine was evaporated to dryness with concentrated hydrochloric acid to convert the ester into hydantoin, and the benzal derivative was prepared as before.

The analytical procedures included the Kjeldahl-Gunning method for nitrogen and Folin's methods for urea and creatinine. Blank experiments with hydantoin showed that this substance is not attacked by Folin's urea method.

In the experiments with rabbits the bladder was emptied by pressure at the same hour daily. The substances, when fed, were dissolved in water and introduced through a gastric sound. In the experiments with dogs the animals were catheterized at reg-

⁵ Harries and Weiss: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 3418, 1900.

⁶ Wheeler and Hoffman: *Amer. Chem. Journ.*, xlv, p. 368, 1911.

ular twenty-four-hour intervals. Here the substances fed were mixed with the food.

EXPERIMENTS WITH HYDANTOIN.

Rabbit. I. Diet, 300 grams of carrots daily. This was completely consumed except on the day of the hydantoin administration when the animal ate only 270 grams. No toxic effects of any sort were noted. The protocol follows:

Rabbit; weight. 1.8 kgms.

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH ₃ N	UREA + NH ₃ -N TOTAL N	N NOT UREA + NH ₃ N	REMARKS
			grams	gram	per cent	gram	
1	250	1.012	0.876	0.768	87.6	0.108	
2	206	1.012	0.918	0.750	80.1	0.168	
3	245	1.015	0.900	0.756	84.0	0.144	
4	145	1.026	1.308	0.738	56.4	0.570	{ 1.5 gm. hydantoin = 0.42 gram N, in traperitoneally.
5	220	1.016	0.696	0.600	86.4	0.096	
6	216	1.024	0.690	0.630	90.1	0.060	

About 0.3 gram of benzalhydantoin, after purification by recrystallization from alcohol, was obtained from the urine of the fourth day. The benzalhydantoin isolated melted at 217° and when mixed with a pure synthetic sample did not alter the melting point of the latter.

No increase in the urea + ammonia nitrogen on the day of the injection was observed, although the increase in the elimination of total nitrogen excreted accounted for all the nitrogen administered as hydantoin. This fact, together with the identification of hydantoin in the urine, indicates that hydantoin is unaltered in its passage through the body.

Another experiment with the same animal a few days later, in which the same amount of hydantoin was administered *per os*, gave similar results.

Rabbit. II. Diet, 300 grams of carrots. On the day on which

the hydantoin was fed, the animal consumed the full daily ration. No toxic symptoms of any sort were noted. The protocol follows:

Rabbit; weight, 1.64 kgms.

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH ₃ N	UREA + NH ₃ -N TOTAL N	N NOT UREA + NH ₃ N	REMARKS
	cc.		gram	gram	per cent	gram	
1	275	1.015	0.900	0.786	87.3	0.114	
2	240	1.018	0.708	0.642	90.6	0.066	
3	150	1.016	0.624	0.558	89.4	0.066	
4	205	1.021	0.918	0.576	62.7	0.342	{ 1.5 gm. hydantoin = 0.42 gram N, per os.
5	280	1.019	0.726	0.570	79.1	0.156	
6	160	1.020	0.600	0.516	86.0	0.084	
7	160	1.025	0.570	0.474	83.1	0.096	

From the urine of the experimental day a small amount of benzalhydantoin melting at 218° was obtained.

Dog. A female was fed on a constant daily diet of 200 grams of lean meat, 50 grams of lard, 30 grams of sugar, 5 grams of bone ash, 2 grams of salt and 250 cc. of water with a total nitrogen content of 6.84 grams. The hydantoin was dissolved in the water of the diet. The animal ate eagerly on the experimental day as at other times.

Dog A; weight, 12.4 kgms.

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH ₃ N	UREA + NH ₃ -N TOTAL N	N NOT UREA + NH ₃ N	CREATININE	REMARKS
	cc.		grams	grams	per cent	grams	gram	
1	160	1.055	8.45	7.63	89.8	0.82	0.279	
2	180	1.042	6.69	6.24	93.3	0.45	0.294	
3	300	1.025	6.38	5.80	90.9	0.58	0.294	
4	300	1.030	6.87	5.67	82.6	1.20	0.298	{ 2.5 gm. hy- dantoin = 0.7 gm. N, with food.
5	320	1.024	5.99	5.39	90.0	0.60	0.306	
6	350	1.023	6.12	5.44	88.8	0.68	0.284	
7	300	1.028	6.37	5.76	90.4	0.61	0.297	

From the urine of the experimental day 3.3 grams of benzalhydantoin were obtained corresponding to 1.7 grams of hydantoin ($= 0.476$ gram N) in the day's urine. This was purified by solution in potassium hydroxide and reprecipitation with acid.

Cat. A cat weighing approximately 4 kgms. received 3 grams of hydantoin mixed with raw meat. The urine of the next twenty-four hours was collected and examined for the presence of hydantoin as above. The benzalhydantoin obtained melted at 214° and after purification weighed 1.7 grams.

A nitrogen determination (Kjeldahl) on the mixed products obtained in this and the three preceding experiments gave the following results:

	Calculated for $C_{10}H_8N_2O_2$:	Found:
N.....	14.92 per cent.	14.59 per cent.

In all our experiments the recovery of the administered hydantoin from the urine leaves no doubt as to its absorption. The increase in the total nitrogen of the urine on the experimental day also points to the same conclusion. In no case was the urea + ammonia nitrogen output increased, the difference between the total and urea + ammonia nitrogen nearly always approaching the value of the nitrogen administered as hydantoin. In the rabbit II, there was observed a slight lag, part of the hydantoin probably being eliminated on the day after the administration. No effect on the creatinine elimination was observed in the dog.

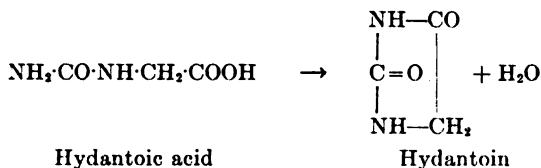
Hydantoin appears to be without influence on nitrogenous metabolism and is not destroyed or changed by the organism. No

toxicity attributable to the group $\begin{array}{c} \text{NH—} \\ | \\ \text{C} = \text{O} \\ | \\ \text{NH—} \end{array}$ as alleged by Lusini was observed.

EXPERIMENTS WITH ETHYL HYDANTOATE.

In order to ascertain whether the inability of the organism to break down hydantoin was due to the cyclic structure, experiments were conducted with the ethyl ester of hydantoic acid. This acid is converted to hydantoin with the loss of a molecule of water

and bears the same relation to hydantoin that creatine bears to creatinine.



Hydantoic acid may also be considered as a uramino acid, uraminoacetic acid. Koehne,⁷ working with the ethyl ester of the homologous uraminoformic or allophanic acid, found that it disappeared in the body.

Rabbit 1. Diet, 300 grams of carrots daily. On the experimental day the animal showed no unusual symptoms and resumed eating immediately after the feeding of the ester. The protocol follows:

Rabbit; weight, 1.7 kgms.

DAY	VOLUME	SPECIFIC GRAVITY	CREATININE	TOTAL N	UREA + NH ₃ N	UREA + NH ₃ N TOTAL N	N NOT UREA + NH ₃ N	REMARKS
	cc.		gram	gram	gram	per cent	gram	
1	220	1.011	0.055	0.810	0.729	90.0	0.091	2 gm. hydantoic acid ester = 0.38 gram N, per os.
2	220	1.012	0.056	0.443	0.347	78.3	0.096	
3	200	1.013	0.047	0.443	0.396	89.8	0.047	
4	295	1.017	0.071	0.845	0.420	49.6	0.425	
5	220	1.016	0.063	0.458	0.347	73.5	0.111	
6	270	1.014	0.061	0.414	0.369	89.1	0.045	

From the urine of the experimental day, benzalhydantoin was prepared in the usual manner. An amount equivalent to 0.67 gram of the ester (= 0.13 gram N.) was obtained. The benzalhydantoin melted at 218° and did not change the melting point of the pure synthetic substance when mixed with it. A nitrogen (Kjeldahl) determination gave the following results:

	Calculated for C ₁₀ H ₈ N ₂ O ₂ :	Found:
N	14.92 per cent.	14.67 per cent.

⁷ Koehne: Inaugural Dissertation, Rostock, 1894, p. 17.

Rabbit 2. Diet, 300 grams of carrots daily. The animal appeared normal on the day of the injection and ate as usual. The protocol follows:

Rabbit; weight, 1.5 kgs.

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	CREATININE	REMARKS
	cc.		grams	gram	
1	95	1.026	1.172	0.103	{ 1.5 grams hydantoic acid ester = 0.286 gram N, subcutaneously.
2	180	1.020	0.702	0.070	
3	220	1.013	0.611	0.078	
4	210	1.013	0.878	0.081	
5	240	1.012	0.513	0.084	
6	240	1.012	0.374	0.076	
7	230	1.016	0.444	0.073	

From the urine of the experimental day a small amount of benzalhydantoin was prepared which melted at 217° and did not affect the melting point of the pure synthetic substance.

Rabbit 3. Diet, 300 grams of carrots and 30 grams of oats daily. On the day of the injection only 270 grams of carrots were eaten. No abnormal symptoms were noted.

Rabbit; weight, 2.34 kgs.

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH ₃	UREA + NH ₃ - TOTAL N	N NOT UREA + NH ₃	CREATININE	REMARKS
	cc.		grams	grams	per cent	gram	gram	
1	265	1.010	1.20	1.020	85.0	0.180	0.122	{ 2 gm. hydantoic acid ester = 0.38 grams N. intraperitoneally.
2	220	1.013	0.855	0.716	82.9	0.139	0.092	
3	220	1.012	1.018	0.840	83.3	0.180	0.110	
4	260	1.015	1.476	1.035	70.3	0.441	0.145	
5	185	1.015	0.996	0.852	85.5	0.144	0.123	
6	160	1.015	0.990	0.900	90.9	0.090	0.135	
7	125	1.018	0.945	0.810	85.7	0.135	0.081	

From the urine of the experimental day benzalhydantoin was prepared and purified by solution in potassium hydroxide and reprecipitation with acid. It weighed 0.75 gram = 0.58 gram ester

in the day's urine. A nitrogen (Kjeldahl) determination gave the following results.

	Calculated for $C_{10}H_8N_2O_2$:	Found:
N.....	14.92 per cent.	14.88 per cent.

Dog. A female received a standard daily diet (see experiments on hydantoin). The food was eaten as usual on the experimental day.

Dog A; weight, 12.6 kgs.

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH_3 N	UREA + NH_3 -N TOTAL N	N NOT UREA + NH_3 N	CREATININE	REMARKS
	cc.		grams	grams	per cent	grams	gram	
1	175	1.040	6.61	6.03	91.2	0.58	0.349	
2	350	1.026	6.60	6.03	91.3	0.57	0.335	3 gm. hydantoic acid ester = 0.67 gm. N in food.
3	380	1.029	6.59	5.93	90.0	0.66	0.324	
4	300	1.028	7.84	6.25	85.1	1.00	0.326	
5	290	1.033	6.25	5.67	90.8	0.58	0.335	2 gm. ester = 0.38 gm. N in food. See note in the text.
6	315	1.026	6.53	5.92	90.7	0.61	0.324	
7	290	1.034	6.44	5.55	86.1	0.89	0.332	

On the seventh day the animal received a second dose of 2 grams of the ester, but refused to eat all the food. The animal was stuffed, and some material was lost. Hence the urine data on that day cannot be compared with those of the previous days. Taken as an isolated experiment, however, the results of the seventh day show the important point that the urea + ammonia nitrogen does not maintain its fairly uniform proportion as on other days but is decreased, indicating excretion of nitrogen in some form other than urea.

Small amounts of benzalhydantoin were prepared from the urine of the experimental days. The material was darker than that obtained in previous experiments and melted at 210–212°. A nitrogen (Kjeldahl) determination gave the following results:

	Calculated for $C_{10}H_8N_2O_2$:	Found:
N.....	14.92 per cent.	14.41 per cent.

These experiments all indicate that in the dog and the rabbit the hydantoin acid ester is not affected by the metabolic processes of the body. No marked increase of urea and ammonia nitrogen was observed. A hydantoin derivative was always isolated from the urine, indicating the presence of the unaltered substance administered. The creatinine output of the urine was not affected. The very slight increase observed in the experiments, especially with rabbit 2, may be accounted for by the fact that the ester itself gives a slight color with Jaffé's picric acid test as it is applied in the colorimeter.

SUMMARY.

1. After administration of hydantoin, the compound can be recovered from the urine in the form of an insoluble benzalhydantoin. This method is not quantitative, but serves to identify the material.

2. No toxic effects were observed to follow the administration of hydantoin. This is in opposition to Lusini's theory of the



toxicity of $\text{C}=\text{O}$ groups.



3. Hydantoin acid, of which hydantoin is the cyclic anhydride, is not destroyed in metabolism when it is administered as the ethyl ester, but can likewise be recovered by forming the insoluble benzalhydantoin.

4. The hydantoin nucleus is not destroyed in the organism of the cat, rabbit or dog.

These studies were undertaken at the suggestion of Professor Lafayette B. Mendel. We desire to acknowledge our indebtedness to Professor Treat B. Johnson for aid in the synthesis of the compounds employed.

THE RACEMIZATION OF PROTEINS AND THEIR DERIVATIVES RESULTING FROM TAUTOMERIC CHANGE.

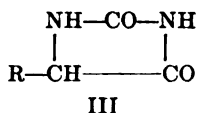
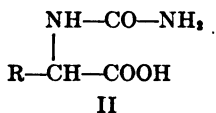
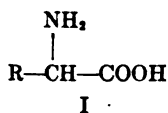
PART I.

By H. D. DAKIN.

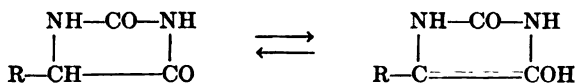
(From the Herter Laboratory, 819 Madison Avenue, New York.)

(Received for publication October 21, 1912.)

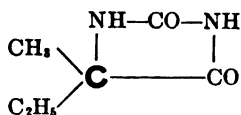
The writer has shown¹ that alkali salts derived from optically active hydantoins (III), prepared from active α -amino-acids (I), undergo spontaneous racemization at room temperature, while the uramido acids (II), intermediate products in the preparation of the hydantoins, like the amino-acids themselves undergo no such change.



The racemization of the hydantoin salts was ascribed to tautomeric change of the keto-enol type, represented as follows:



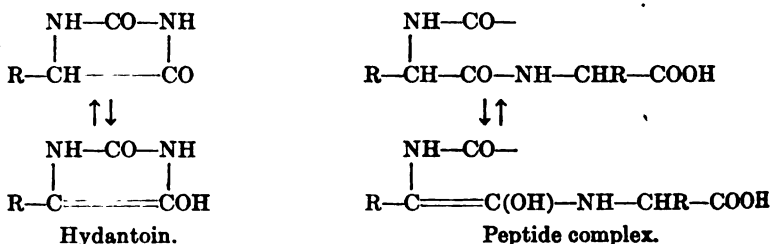
The formation of salts of the enol type, even to a small extent, would necessarily be followed by racemization since the asymmetry of the α -carbon atom is abolished. The view that racemization was due to the formation of salts of the enol type was further confirmed by the observation that *d*-methylethylhydantoin,



¹ *Amer. Chem. Journ.*, xlv, p. 48, 1910.

a substance in which no labile hydrogen atom is present, retained its optical activity indefinitely under conditions which resulted in complete racemization in the case of hydantoin salts capable of keto-enol isomerism.

A consideration of the changes involved in the racemization of these amino-acid derivatives led to the belief that similar transformations would be found to occur in the proteins and polypeptides.² For while salts of free amino-acids cannot exhibit keto-enol isomerism, the opposite may be true of certain of the groups in peptide structures. The following formulae comparing the hydantoin and peptide groupings, make this clear:



A consideration of the above formulae would indicate that, of the two amino-acid groups present in the above peptide complex, only the one containing the $-\text{CH}-\text{CO}-$ group could exhibit keto-enol tautomerism and hence racemization; while the terminal amino-acid group containing a free carboxyl group would be unchanged.

This type of change apparently corresponds closely to what takes place when a protein is digested at low temperature with dilute alkali. Kossel and Weiss have shown that proteins as widely different as gelatin and clupeine show a rapid decline in optical activity when digested at low temperatures with dilute alkali, but in no case is optical activity completely abolished. Kossel and Weiss further show that on hydrolyzing the racemized protamines inactive arginine was obtained, while gelatin gave a variety of products from which on hydrolysis inactive histidine, arginine, ornithine and some inactive lysine were obtained.³

² The racemization changes described in this paper are all effected at low temperatures and have nothing in common with the racemization of amino-acids, etc., at high temperatures when heated with caustic alkalis.

³ *Zeitschr. f. physiol. Chem.*, lix, p. 492, 1909; lx, p. 311, 1909; lxxviii, p. 165, 1910.

Kossel and Weiss regard the phenomenon essentially as one of protein racemization, somewhat complicated by simultaneous hydrolysis due to the action of the alkali. Moreover, they came to the conclusion that certain amino-acid groups, *e.g.*, arginine, undergo racemization much more readily when intramolecularly bound than when free. It will be noticed that these conclusions harmonize excellently with the writer's hypothesis and, with Professor Kossel's permission, the writer is attempting to obtain further insight into the mechanism of the action of alkali on proteins.

Preliminary experiments upon the action of dilute alkali on gelatin show that certain of the amino-acid groups are readily and completely racemized, while others retain the optical activity unimpaired, while some appear to occupy an intermediate position.

The optical properties of some of the amino-acids obtained by hydrolyzing the products resulting from the action of caustic soda upon gelatin, are recorded below.

Four hundred gram portions of moist gelatin were dissolved in 4 liters of water and then 200 cc. of 33 per cent caustic soda added. The initial rotation was approximately -13.5° and sank to -6.28° after twenty-four hours, -3.39 after four days and finally became practically constant at -2.32° after fifteen days. The mixture containing ammonia, free amino-acids, peptones and protein was completely hydrolyzed with acids and the mono-amino-acids examined by Fischer's ester method, while the bases were isolated by the methods of Kossel and Kutscher.

Glycine was separated as the ethyl ester hydrochloride. The glycine was, of course, inactive. Yield, about 17.5 per cent.

Alanine was obtained in the form of mixtures containing from 25 to 60 per cent of the active form, the balance being inactive. Although some racemization takes place during the separation of the alanine, the proportion of inactive substance is rather high and probably indicates some racemization prior to hydrolysis.

Leucine. Large quantities (about 1.5 per cent) of pure inactive leucine were readily obtained on recrystallizing the "leucine fraction."

ANALYSIS: 0.1458 gram gave 0.2950 gram CO_2 and 0.1327 gram H_2O .
0.1937 gram gave $\text{NH}_3 = 0.0209$ gram N.

	Found:	Calculated for $\text{C}_6\text{H}_{10}\text{O}_2\text{N}$:
C.....	55.2	55.0
H.....	10.1	9.9
N.....	10.8	10.7

All of the leucine that was obtained in a satisfactorily pure state was optically inactive in both aqueous and hydrochloric acid solutions.

A portion of the inactive leucine was converted into α -uramido-isobutylacetic acid by means of potassium cyanate. On recrystallization from boiling water the substance was obtained in the form of platelets, m.p., 203° – 204° .

The mother liquors from the leucine fractions contained some alanine and were feebly laevorotatory in aqueous solution and dextrorotatory in hydrochloric acid. Other amino-acids were apparently present.

Proline. A yield of proline was obtained about one-half larger than that recorded by Fischer, Plimmer and Levene. Its rotation in aqueous solution indicated that it contained about 21 per cent of the laevo variety. Since proline is largely racemized in the process of isolation, it is probable that little or no racemization occurred before hydrolysis.

The proline was characterized by conversion into the copper salt and into the phenyl-hydantoin.

Aspartic acid. The aspartic acid isolated in the usual way was completely inactive. The yield was 0.6 per cent. No indications were obtained of any active aspartic acid.

Glutamic acid was separated as hydrochloride. Its rotation indicated that no racemization had occurred.

ROTATION: $\alpha = +3.92^\circ$; $l = 2$ dm.; $c = 6.55$.

$$[\alpha]_D^{20} = +29.9^\circ$$

Phenylalanine was separated as hydrochloride. A 2 per cent solution was completely inactive. A yield of slightly over 1 per cent was obtained, or more than twice that previously recorded.

ANALYSIS: 0.1600 gram gave 0.3845 gram CO₂ and 0.0986 gram H₂O.

	Found:	Calculated for C ₆ H ₁₁ O ₂ N:
C.....	65.5	65.5
H.....	6.8	6.7

Histidine was isolated by means of the phosphotungstate, silver compound and picrolonate. A 3.5 per cent solution of the base dissolved in hydrochloric acid showed a doubtful rotation of -0.08° in a 1 dm. tube. Racemization was evidently practically complete.

Arginine was separated according to Kossel's methods and was inactive. The picrolonate melted at 241° - 242° and contained 25.4 per cent N. (Theory = 25.6.)

Lysine and ornithine were precipitated as phosphotungstates after the removal of histidine and arginine. The bases were converted into picrates and recrystallized from hot water. The less soluble lysine picrate was readily obtained and on decomposition with hydrochloric acid, gave dextrorotatory lysine dihydrochloride.

ROTATION: $\alpha = +2.60^\circ$; $l = 2$ dm.; $c = 9.5$.

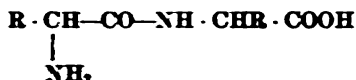
$$[\alpha]_D^{20} = +13.7^\circ$$

Discussion of results. The foregoing experiments show that gelatin, when digested with dilute alkali and subsequently hydrolyzed with acids, yields inactive leucine, aspartic acid, arginine, histidine and phenylalanine, while proline, glutamic acid and lysine are obtained in the optically active forms together with part of the alanine. It is certainly a striking fact that substances so closely allied as aspartic and glutamic acids or lysine and arginine should exhibit such diametrically opposite behavior.

The interpretation of these results is necessarily difficult. But in view of the probability of racemization being due to keto-enol, tautomeric change taking place in alkaline solution, and since such racemization can only occur in amino-acid groups in which the carboxyl group attached to the asymmetric carbon atom is in union with other groups (see p. 358), the inference appears probable that none of the carboxyl groups in leucine, aspartic acid, arginine, histidine or phenylalanine are free in gelatin. On the other hand, since glutamic acid and lysine and possibly alanine are obtained in

active forms, it is possible that some of their carboxyl groups may be free or, in other words, that they *may* occupy terminal positions in the peptide chains. On the other hand, it is possible that these amino-acids are rapidly liberated in the free state by the hydrolytic action of the alkali and so escape racemization. Further work will be necessary to decide this point.

Since simple dipeptides containing a free amino group:



or peptides such as prolylglycine, apparently do not undergo ready racemization with dilute alkali it appears likely that the conditions necessary for racemization of an amino-acid group require the attachment of other groups to both amino and carboxyl radicle.

In order to fully elucidate the mechanism of the changes involved in the action of dilute alkali on proteins, much additional work is needed and it will be necessary to make experiments with peptides of known structure. It would appear that the study may be of service in throwing some light on the relative positions of some of the groups in protein and peptide complexes and, in addition, may furnish information as to the structure of protein salts. The investigation is being continued.

A NEW METHOD FOR THE (COLORIMETRIC) DETERMINATION OF URIC ACID IN URINE.

BY OTTO FOLIN AND A. B. MACALLUM, Jr.

(From the Biochemical Laboratory of the Harvard Medical School, Boston.)

(Received for publication, October 26, 1912.)

In a previous number of this *Journal*¹ we published a preliminary paper on the color produced by the action of phosphotungstic acid on uric acid and certain other substances and we outlined a method for the determination of uric acid by means of that color reaction. Inasmuch as the ordinary method for preparing phosphotungstic acid solutions for this reaction (boiling 20 parts of sodium tungstate with 10 parts of 20 per cent phosphoric acid) gave reagents which had little or no action on uric acid solutions, we began to suspect that the active ingredient was some impurity, variable amounts of which might be present in the sodium tungstate. The same impurity might have been present in molybdenum compounds since phosphomolybdic acid also gave variable and not very strong color reactions with uric acid. A more systematic investigation² soon showed that the amount of active material contained in the phosphotungstic and phosphomolybdic acid solutions was determined by the conditions under which the solutions were prepared and was particularly dependent upon the proportion of phosphoric acid used. It was further found that reagents containing phosphomolybdic acid reacted with all phenols as well as with uric acid while the phosphotungstic acid reagents gave the blue color only with polyphenols and with uric acid. This point of difference is of considerable importance in respect to a colorimetric determination of uric acid in urine. By using the phosphotungstic acid reagent the phenol reaction is in a large measure eliminated. Our "uric acid reagent" accordingly is a

¹ This *Journal*, xi, p. 265, 1912.

² Folin and Denis: this *Journal*, xii, p. 239, 1912.

phosphotungstic acid prepared by boiling 100 grams of sodium tungstate with 80 cc. of 85 per cent phosphoric acid and 750 cc. of water for a couple of hours and then diluting to 1 liter.

Since the publication of our first paper E. Riegler has published a paper in the *Zeitschrift für analytische Chemie*³ in which he describes a colorimetric method for the determination of uric acid based on the use of phosphomolybdic acid. Although Riegler's new method is quite different from ours, it would seem best to specifically call attention here to the fact that our first paper on the subject was published in the April number of this *Journal* and a second paper which appeared in the August number was received by the Editor of the *Journal* on June 29. Riegler's paper appeared in the seventh and eighth double number of the above mentioned *Zeitschrift* which was evidently published some time in June and did not reach us until July. Riegler evidently has not made any particular study of the color reaction involved or of the conditions under which the maximum color can be obtained. He uses phosphomolybdic acid for producing the blue color, and the amount of color which he obtains from a given quantity of uric acid is evidently not nearly as deep as the color which we secure, since his standard color is that given by 1 mgm. of uric acid when diluted to 10 cc., while ours is the same amount of uric acid diluted to 100 cc. The color which we obtain with 1 mgm. of uric acid by means of our reagent would be too dark to transmit any light if diluted to only 10 cc. Yet unlike Riegler's reagent, ours gives no color with simple phenols or with protein materials. These advantages are of course the result of our having prepared phosphotungstic acid reagents specifically with reference to their chromophoric value, while Riegler uses phosphomolybdic acid prepared for ordinary precipitating purposes.

In our earlier work we precipitated the uric acid in urine by a modification of Salkowski's silver method (using silver sulphate instead of nitrate because nitrates interfere with the color reaction). The precipitation, washing, etc., was all done in centrifugal tubes and the final blue solutions were then rinsed into volumetric flasks and made up to volume before reading off the color.

After we had obtained a reagent which did not react with protein materials or other monophenol derivatives we hoped to be able to make the color directly on the urine without any preliminary treatment whatever. The uric acid values so obtained continue however to be high as compared with the uric acid obtained by the older methods,⁴ and we were forced to recognize the fact

³ *Zeitschr. f. anal. Chem.*, li, p. 466, 1912.

⁴ For exact figures indicating the errors which would come in because of these polyphenol derivatives, see column 4, p. 369.

pointed out long ago by Baumann that urines contain di- and poly-phenol acids in addition to the more abundant monophenol derivatives. Having obtained a clear idea as to the probable nature of the substances which were responsible for the extra color produced by the uric acid reagent in urine, we have now worked out a new method for the determination of uric acid which is materially simpler than the method outlined in our first paper.

This method is described in some detail below.

From 2 to 5 cc. of urine (the amount depending on the specific gravity) are measured into a 100 cc. beaker and after adding a drop of saturated oxalic acid solution the whole is evaporated to dryness on the water bath or over a hot iron plate or even over a free flame. This evaporation takes only a few minutes, but it should be continued until the upper parts of the beaker are dry. To the dry, cool residue are added 10–15 cc. of a mixture consisting of 2 parts of pure, dry ether (*i.e.*, ether distilled over sodium) and 1 part of pure methyl alcohol. After standing for about five minutes the solution is removed by careful decantation and another 10 cc. of the alcohol-ether mixture are added to the residue, allowed to settle and decanted. The alcohol-ether dissolves out the phenol acids and leaves the uric acid.

With regard to this treatment of the evaporated urine residue for the removal of other substances than uric acid which give a blue color with the reagent, it should be said that two very slight sources of error are inherent in the process. In the presence of considerable solid residues, soluble phenol acids, as, for example, tannic acid, are not to be perfectly removed by the treatment outlined above. Nor is uric acid absolutely insoluble in the organic solvent under the conditions of the experiment. These two sources of error are however very slight indeed and as they happen to influence the result in opposite directions they do not appreciably affect the analytical results. For urine work ordinary 90 per cent ethyl alcohol might, as a matter of fact, be substituted for the ether-alcohol mixture recommended above but, since we have satisfied ourselves that uric acid is rather more soluble in it than in the ether-alcohol mixture, we prefer the latter.

To the washed residue in the bottom of the beaker is next added water (5–10 cc.) and a drop of saturated sodium carbonate solution, and the mixture is shaken or stirred so as to secure complete

solution of the uric acid. To the solution is finally added, first, 2 cc. of the uric acid reagent and then 20 cc. of saturated sodium carbonate solution. The resulting blue solution is transferred to a 100 cc. measuring flask, diluted with water up to the mark and the intensity of the color is determined by means of a Duboscq colorimeter. The standard solution for comparison is obtained by treating 1 mgm. of uric acid in lithium carbonate solution with 5-10 cc. of water, 2 cc. of the uric acid reagent and 20 cc. of sodium carbonate solution, and the whole made up to 100 cc. in a volumetric flask.

The uric acid reagent and carbonate solution should be added as nearly simultaneously as practicable to both the unknown and the standard because the solutions obtained deteriorate rather rapidly (sufficiently rapidly for example to produce an unmistakable difference in color in fifteen minutes). This deterioration is of no consequence to the result if the solutions are made at about the same time, for the known and the unknown will deteriorate at practically the same rate. It is therefore perfectly feasible to make several uric acid determinations by the help of the same standard solutions provided the uric acid reagent has been added to all of them at about the same time (*i.e.*, within three to five minutes).

As stated above, the color obtained from 1 mgm. of uric acid is used as a standard. Unfortunately uric acid solutions do not keep very well though when properly made they last for about a week, certainly for several days. To obtain such stable uric acid solutions it is necessary to add only just enough lithium carbonate to dissolve the uric acid in the course of about an hour. A 0.4 per cent lithium carbonate solution is very convenient for the purpose. Each cubic centimeter of such a solution dissolves 10 mgm. of uric acid. For a standard solution we therefore weigh out 250 mgm. of Kahlbaum's uric acid, transfer it to a 250 cc. volumetric flask by means of 25-50 cc. of water, then add 25 cc. of the lithium carbonate solution and shake at intervals for an hour before diluting with water.

The preparation of such a standard uric acid solution once a week is not much of a task if in the course of that week a number of uric acid determinations are to be made. A standard solution which remains reliable only for a week is however far from an ideal standard.

We have devoted a great deal of time trying to find a more permanent standard, for it would manifestly be a great advantage to have it always on hand for occasional uric acid determinations.

Riegler adopted colored glasses for the purpose, but so far we have been unable to find any which are serviceable, because they transmit too much or too little light. We have also investigated a large number of substances which give blue solutions, including the aniline dyes, but here again they are entirely too bright to be satisfactory with the ordinary Duboscq colorimeter.

We have also experimented with a large number of substances (polyphenol derivatives) which give the blue color with the uric acid reagent but curiously enough nearly all give blue solutions which are less bright than the blue given by uric acid, besides having a more greenish shade.

It therefore seems almost hopeless to get any standard solution of keeping quality capable of giving exactly the same color as that obtained with uric acid. A good, though by no means ideal standard solution, was finally found in the uric acid reagent itself. It was found that an excess of the reagent used with a minute quantity of uric acid gives the same color as is obtained when an excess of uric acid is added to a minute quantity of the uric acid reagent; and as the latter seems to keep indefinitely, a part of it can be set aside and standardized against a known uric acid solution and will then be found to be very serviceable for occasional uric acid determinations.

When employed as a standard the uric acid reagent is used as follows: A small pinch (a few milligrams) of uric acid is thrown into a 100 cc. measuring flask and is dissolved by the addition of 20 cc. of saturated sodium carbonate solution. (No attention need be paid to the rapidity or completeness with which the uric acid dissolves.) To this solution is then added 1 cc. of the uric acid reagent or a corresponding quantity of a uric acid reagent previously diluted with water. The blue solution is diluted to 100 cc. and its color value is determined by comparison with the color given by 1 mgm. of uric acid when treated in the usual manner with an excess (2 cc.) of the uric acid reagent.

Setting the prism of the known uric acid solution at 2—mm., it will be found that 1 cc. of the reagent treated with an excess of uric acid as described will read somewhere between 9 and 12 mm. Suppose it reads at the latter figure; then setting it at 12 when

using it as a standard for the determination of uric acid in an unknown solution or in urine is the same as using 1 mgm. of uric acid and setting the standard at 20. To illustrate: If an unknown urine against this new standard gave a color of the same intensity as the standard named, it would contain $\frac{4}{3}$ or 1.666+ mgm. of uric acid in the volume of urine taken for analysis; if the unknown read 11 mm. with the standard at 12, it would contain $\frac{4}{3}$ or 1.82 mgm. of uric acid.⁵ The chief drawback encountered in using the uric acid reagent as a standard is that the color produced fades more rapidly than the color produced when the reaction is made in the usual way with an excess of the reagent. It is therefore necessary to make the reaction as nearly simultaneously as possible in the unknown and in the standard and then to read the color promptly, *i.e.*, within five minutes. For each unknown to be determined a fresh reaction must be made with the standard. When a series of uric acid determinations are to be made it is therefore in most cases best to use uric acid rather than the uric acid reagent as a standard.

The uric acid values recorded below for twenty normal urines show that the new colorimetric method gives substantially the same figures as are obtained by the Folin-Shaffer modification of Hopkin's method. The figures given in the fourth column represent the uric acid plus all other substances in urine which give a blue color with our uric acid reagent. As indicated in the table, we regard these as being chiefly polyphenols.

Since the reaction for uric acid which is here used as a basis for its determination is more delicate than any other known reaction and is perfectly adaptable for quantitative work, it is clear that it ought to prove eminently suitable for the determination of such small quantities of uric acid as may exist in blood. A new method for the determination of uric acid in blood, based on the principles outlined in this paper, will be described in the next number of this *Journal*.

⁵ It doubtless will occur to some that it would be more convenient to dilute the uric acid reagent so that a suitable volume should have the same chromophoric value as the solution obtained from 1 mgm. of uric acid. There is however nothing to be gained by such a procedure. It is just as easy to set the standard somewhere between 9 and 12 as at 20. Moreover, the more concentrated color corresponding to 1 cc. of the uric acid reagent fades less rapidly than do weaker solutions.

Uric acid in urine (grams per liter).

NO.	FOLIN-SHAFFER METHOD	COLORIMETRIC METHOD	URIC ACID AND POLYPHENOLS	SPECIFIC GRAVITY OF URINE
1	0.16	0.19	0.26	1.012
2	0.49	0.50	0.59	1.027
3	0.50	0.49	0.67	1.027
4	0.41	0.41	0.60	1.017
5	0.49	0.49	0.60	1.027
6	0.59	0.58	0.81	1.027
7	0.57	0.56	0.72	1.030
8	0.41	0.40	0.54	1.027
9	0.42	0.41	0.51	1.027
10	0.41	0.43	0.54	1.022
11	0.13	0.16	0.40	1.012
12	0.36	0.36	0.52	1.022
13	0.67	0.67	0.75	1.027
14	0.28	0.28	0.45	1.019
15	0.23	0.23	0.41	1.018
16	0.31	0.32	0.39	1.021
17	0.78	0.74	0.87	1.030
18	0.60	0.62	0.75	1.029
19	0.74	0.74	1.01	1.028
20	0.51	0.50	0.63	1.025



THE METABOLISM OF ENDOGENOUS AND EXOGENOUS PURINES IN THE MONKEY.¹

BY ANDREW HUNTER AND MAURICE H. GIVENS.

(From the Department of Physiology and Biochemistry, Cornell University, Medical College, Ithaca, N. Y.)

(Received for publication, November 1, 1912.)

The introduction by Wiechowski² of an accurate method for the determination of allantoin led very quickly to the recognition of that substance as a constant constituent of the urine of mammals, to be included with uric acid and the purine bases among the regular products of endogenous purine metabolism. It appears practically certain that in no mammalian urine is any one of these three stages in the progressive catabolism of the purine nucleus entirely unrepresented. As for the relative importance of each, it is found to vary with the species. Some animals excrete uric acid in greater abundance than purine bases,³ while for others the reverse is true. In most cases the rôle of these two fractions combined is greatly subordinate to that of allantoin. Thus in the dog⁴ allantoin accounts for 93 to 97 per cent of the total purine nitrogen eliminated, and a ratio of the same order has been established for the cat,⁵ the rabbit,⁵ the pig⁶ and the coyote.⁷ In another group of animals,

¹ The experimental matter contained in this paper was presented in part at the Joint Meeting of the American Society of Biological Chemists and the Biological Section of the American Chemical Society, Washington, December, 1911, and in part at the Eighth International Congress of Applied Chemistry, New York, September, 1912.

² Wiechowski: *Beitr. z. chem. Physiol.*, xi, p. 109, 1908.

³ This is known to be true for the horse (Schittenhelm and Bendix: *Zeitschr. f. physiol. Chem.*, xlviii, p. 140, 1906) and the pig (Schittenhelm and Bendix: *loc. cit.*; Schittenhelm: *Zeitschr. f. physiol. Chem.*, lxvi, p. 53, 1910; Mendel and Lyman: this *Journal*, viii, p. 115, 1910).

⁴ Wiechowski: *loc. cit.*; Schittenhelm: *Zeitschr. f. physiol. Chem.*, lxii, p. 80, 1909; Hirokawa: *Biochem. Zeitschr.*, xxvi, p. 441, 1910.

⁵ Wiechowski: *loc. cit.*

⁶ Schittenhelm: *loc. cit.*, 1910.

⁷ Hunter and Givens: this *Journal*, viii, p. 449, 1910.

exemplified by the horse⁸ (and as will appear, the monkey), the predominance of allantoin is less pronounced. One species, namely man, occupies in this respect an altogether exceptional position. Human urine is characterized by its high content of uric acid; bases are present in relatively small amounts; while allantoin has been detected in the merest traces.⁹

It is natural to inquire whether the specific peculiarities thus manifested permit of being systematized. Particularly is one tempted to ask whether in this province similarities of physiological reaction correspond with genetic affinities and whether among the variations encountered it is possible to trace any evolutionary sequence. With the purpose of collecting data which may ultimately afford an answer to such questions we have undertaken the systematic examination of a number of mammalian urines not hitherto studied from this point of view.

The present paper deals with one species only, but it is representative of a group to which a special interest attaches, and it has been made the subject of rather more extended observation and experiment than lay in our original plan. The apparently unique behavior of the human organism suggested an early attempt to ascertain the character of the purine-allantoin excretion in man's nearest relatives. Material from the higher apes we have not been fortunate enough to have at our disposal but about two years ago we were able to take up the problem in regard to the monkey. It is true that even at that time data on the subject were not altogether lacking. Wiechowski¹⁰ had examined one sample of monkey urine (species not stated), and had found it to contain as much as 0.1 gram of allantoin in 100 cc.; uric acid was absent; purine bases were apparently not determined. To the general question involved an important contribution had also been made by Wells.¹¹ In a study devoted particularly to the purine enzymes of the tissues of *Macacus rhesus* he demonstrated that the liver of this

⁸ Wiechowski: *Biochem. Zeitschr.*, xix, p. 368, 1909.

⁹ Wiechowski: *loc. cit.*, 1909 and *Biochem. Zeitschr.*, xxv, p. 431, 1910; Schittenhelm and Wiener: *Zeitschr. f. physiol. Chem.*, lxi, p. 283, 1909; Ascher: *Biochem. Zeitschr.*, xxvi, p. 370, 1910; Fairhall and Hawk: *Journ. Amer. Chem. Soc.*, xxxiv, p. 546, 1912.

¹⁰ Wiechowski: *loc. cit.*, 1908.

¹¹ Wells: this *Journal*, vii, p. 171, 1910.

monkey exhibits *in vitro* a high degree of uricolytic power. In spite of this he was unable, either by the Wiechowski method or by the older one of Poduschka,¹² to isolate allantoin from the urine of the animals employed. At the most its presence was rendered probable. Uric acid was encountered on one occasion only, and was then ascribed to the effects of tuberculous disease. The total purine excretion was exceedingly small.

Published observations on the point specially interesting us were, it is seen, not only few in number but rather imperfectly concordant, and we felt justified in pursuing the question independently. In a first communication¹³ we confirmed Wiechowski as to the presence of allantoin in monkey's urine, Wells as to the smallness of the total purine output and both as to the normal absence of uric acid. But the few analyses reported were made on mixed samples obtained at irregular intervals from two monkeys of different species. They possessed therefore little more than a qualitative value. We have since subjected one of the animals to a series of quantitative studies. An abstract of our earlier results had already been published,¹⁴ when a fresh contribution to the subject appeared from the pen of Wiechowski.¹⁵ In this he states the conclusion, fully in accord with our own observations, that in monkeys the principal end product of purine metabolism is allantoin; while he reports in addition the extremely interesting discovery, that this substance is absent from the urine of the chimpanzee. Although our program, as originally planned, is not yet completed, the appearance of Wiechowski's paper leads us to render without further delay a detailed account of some of our experiments.

¹² Poduschka: *Arch. f. exp. Path.*, xliv, p. 59, 1900.

¹³ Hunter and Givens: *Proc. Amer. Soc. Biol. Chem.*, December, 1910, *this Journal*, ix, p. xvi, 1911.

¹⁴ Hunter and Givens: *Proc. Amer. Soc. Biol. Chem.*, December, 1911, *this Journal*, xi, p. xxxix, 1912. Further data, some of which also are incorporated in the present paper, formed the subject of another brief communication: see *Orig. Comm. Eighth Intern. Congr. of Applied Chem.*, xix, p. 149, September, 1912. This was already in print before we became aware of the existence of Wiechowski's recent article.

¹⁵ Wiechowski: *Prager med. Wochenschr.*, 1912, p. 275. The original paper is not within our reach; we quote from an abstract in *Zentralbl. f. Biochem. u. Biophys.*, xiii, p. 661, September, 1912.

SUBJECT AND METHODS.

The subject was an adult female "guenon" monkey (*Cercopithecus callitrichus*),¹⁶ weighing 4.7 to 4.8 kgm., of very active habit and lively though uncertain temper. When under observation it was confined in a metabolism cage of the ordinary type and was maintained upon a diet limited to milk, peanuts and bananas, with the occasional addition of some common salt. Water, as a separate item, was neither given nor apparently desired.

We hoped at first to be able to maintain the animal in uninterrupted nitrogenous equilibrium, but the rather capricious nature of its appetite forced us to abandon the attempt. The diet was at least adequate, the body weight increased rather than diminished during the experiments and except possibly towards the end of the second period of confinement the monkey was in perfect condition.

The oral administration of sodium nucleate and of allantoin was effected by dissolving them in the minimum amount of water and adding the solution to the morning draught of milk. During the day successive portions of the milk ration were offered in the same polished nickel dish; quantitative ingestion of the dose was thus in every case assured.¹⁷ The subcutaneous injections were made from an elevated burette into the loose tissue round the loins. They produced no perceptible local or general effects.

The urine was collected in forty-eight-hour periods. The temper of the animal made the use of the catheter out of the question. Fortunately she could be induced to micturate at any time by transferring her from one cage to another, and by taking advantage of this it was possible to delimit each period's excretion with an error of less than twenty minutes. As the urine was frequently alkaline when passed, it was received in a flask containing acetic acid. Putrefaction was prevented by the liberal use of thymol. Each sample, as collected, was made up along with the cage washings

¹⁶ The species was identified for us by Dr. H. D. Reed of the Department of Vertebrate Zoology in this University. To Dr. Reed we desire to take this opportunity of expressing our indebtedness for the service.

¹⁷ On a few occasions after the administration of sodium nucleate a part of the last portion of milk for the day was refused. This was hydrolyzed with 5 per cent sulphuric acid and tested for purines by the method of Krüger and Schmid. No purines were ever detected.

to a round volume of 1000 cc. All the analyses were commenced immediately.

Uric acid and *purine bases* were determined in a 400 cc. portion. The method employed was that of Krüger and Schmid¹⁸ with these variations, that the use of MnO_2 was omitted, that the filtrate and washings from uric acid (when present) were not allowed to exceed 25 cc. and that the correction for solubility of uric acid was not applied. It follows that the figures under "purine bases" may, in the reported absence of uric acid, and do, in its presence, include a trace of the latter. The error involved is not greater than 1 mgm. for each two-day period.

Allantoin was estimated by the method of Wiechowski. Its application to the urine of our monkey proved to be not altogether a simple matter. In fact in the first form described by its author it yielded us no allantoin whatever. The difficulty lay, as afterwards appeared, partly in the small amount of allantoin actually excreted—less than 0.05 gram daily—and partly in the presence of substances which to all the precipitants employed reacted exactly like allantoin itself. These obstacles were finally overcome by a procedure essentially the same as that employed by Wiechowski with human urine.¹⁹

Two-fifths (400 cc.) of each urine sample, acidified with sulphuric acid, was treated with the requisite amount (about 15 cc.) of 50 per cent phosphotungstic acid. The phosphotungstates were separated on a kieselguhr filter.²⁰ The filtrate was neutralized with sodium hydroxide and treated, after the usual routine, first with 20 per cent basic lead acetate and then with a saturated solution of acetate of silver. Every precipitate, especially the very heavy lead one, was thoroughly washed with cold water and the washings were added to the filtrate. The final bulk of the liquid was consequently very considerable (about 1200 cc.). It was carefully neutralized with sodium hydroxide and subjected to a partial precipitation with 20 per cent mercuric nitrate. 8 cc. of this reagent were all that were required to completely precipitate the allantoin; 5 cc. of the filtrate invariably gave an immediate reaction with one drop of a 0.1 per cent solution of allantoin.

¹⁸ Krüger and Schmid: *Zeitschr. f. physiol. Chem.*, xlv, p. 1, 1905.

¹⁹ Wiechowski: *loc. cit.*, 1909.

²⁰ The kieselguhr filter recommended by Wiechowski was employed not only here, but also in filtering the very fine silver precipitate of a later stage of the process. It proved to be in these and many other situations exceedingly efficient for the retention of fine suspensions; but we are not without suspicion that its use was at least partly responsible for the ash content of our allantoin (see later).

After standing over night the precipitate was collected, suspended in a little water and decomposed in the cold with hydrogen sulphide. The hydrogen sulphide having been expelled and the sulphide filtered off, the filtrate and washings were exactly neutralized and precipitated with Wiechowski's mercuric acetate reagent. The allantoin solution obtained by the decomposition of the mercury precipitate was evaporated to dryness in a tared dish. The residue was carefully washed in the dish with a little cold water, dried at 105° and weighed.

The product thus reckoned as allantoin consisted of practically colorless crystals of typical form. They melted at 231–232° and the melting point was not altered by admixture with pure allantoin.

In spite of these indications of purity the nitrogen content of the crystals was always lower than that calculated for pure allantoin. This we ascertained to be due to the constant presence of slight mineral impurity. This could of course be removed by repeated recrystallization; 0.0232 gram of a thrice crystallized sample yielded 8.19 mgm. of nitrogen, corresponding to 35.3 per cent; calculated, 35.4. But purification in this way was anything but quantitative. Fortunately the error caused by the presence of inorganic material was practically compensated by another which operated in the opposite direction. In washing the crude crystals we inevitably removed not only the bulk of the pigment and other easily soluble contaminations but also a small amount of the allantoin itself. Allantoin in fact, as we have repeatedly noticed, though hardly soluble in cold water when pure, is distinctly so in the presence of the impurities by which it is here accompanied. That the inaccuracies thus involved in our procedure were really of no very great significance, we satisfied ourselves by a number of control experiments.

In these, weighed quantities of allantoin were added to monkey urine of which the allantoin content had been previously determined. All analyses were carried out in duplicate; in one set the determinations were made gravimetrically, as above described, and in the parallel one the mercury precipitates were Kjeldahled. The results of the two sets were nearly identical. Each liter of urine contained, to start with, 28 mgm. of allantoin nitrogen as weighed or 26 according to the nitrogen determination. Of added allantoin there was recovered in the gravimetric series 91–97 per cent and in the volumetric one 89–91 per cent.²¹

In the second series of experiments reported below (table II) the allantoin was not, as in the first, weighed after isolation but was calculated from the nitrogen content of the mercury precipitate. There are doubtless strong objections to the adoption of this as a routine procedure, but we have ample evidence that in the present case at least it was equivalent in its results to the other. This appeared in the control analyses described above. We have moreover many times, after weighing the washed crystals, subjected them along with the collected washings to a Kjeldahl determination. The following exemplify the results:

²¹ Levene and Medigreceanu (*Amer. Journ. of Physiol.*, xxvii, p. 438, 1911) were able to recover from human urine up to 90 per cent of added allantoin.

NUMBER OF URINE	ALLANTOIN BY WEIGHT	CALCULATED FROM TOTAL NITROGEN
	<i>mgm.</i>	<i>mgm.</i>
7	33.0	31.0
9	32.5	34.0
11	36.0	34.5

Apparently the impurities precipitated with the allantoin are practically free from nitrogen. It will be noted, as a further confirmation of this inference, that the "normal" amount of allantoin in table II is the same as in table I.

The *feces*, collected daily, were kept in dilute sulphuric acid and in combined lots, marked off by the administration of carmine, were analyzed for purines by the method of Krüger and Schittenhelm.²²

ANALYTICAL RESULTS.

The first series of observations made was planned to ascertain the normal endogenous excretion of purines and allantoin together with the effect of feeding nucleic acid. The monkey was maintained for forty days upon a daily ration of 200 cc. of whole milk, 200 grams of bananas and 20 grams of peanuts, calculated to contain about 2.09 grams of nitrogen and 418 calories. The urine analyses for the first twelve days were incomplete. The record for the remaining twenty-eight is reproduced in Table I.

The experiments of Table I were followed by a period of six weeks' liberty, during which the monkey enjoyed a fairly promiscuous diet. After being returned to the cage it was placed upon the slightly altered allowance of 150 cc. of milk, 150 grams of bananas and 25 grams of peanuts, equivalent to about 1.91 grams of nitrogen and 363 calories. Table II exhibits the results obtained during fifty-eight days under this régime. Besides a preliminary normal period of two weeks, there are included two more experiments with nucleic acid and a series upon the enteral and parenteral administration of allantoin.

The excretion of endogenous purines and allantoin.

In Table I there occur nine two-day periods and in Table II twenty-one, upon which no foreign substance was administered.

²² Krüger and Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlv, p. 14, 1905.

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In two of these (numbers 38 and 41) the effect of feeding sodium nucleate during the preceding forty-eight hours may still be traced. For five others the record is imperfect. There remain twenty-three "normal" periods, which, since the regular diet could have contained no appreciable quantity either of purines or of allantoin, may be taken to represent the endogenous excretion of the substances we are considering.

TABLE I.

PERIOD	GRAMS NITROGEN IN 48 HOURS					ALLANTOIN N IN PER CENT OF TO- TAL PURINE- ALLANTOIN N	REMARKS
	Total	Allan- toin	Uric acid	Purine bases	Total purine- allan- toin N		
1	3.59		none	0.009			
2	3.81	0.031	none	0.009	0.040	78	
3	3.64	0.031	none				
4	*3.09	0.045	none	0.009	0.054	83	0.5 gram sodium nucleate per os.
5	3.18	0.027	none	0.008	0.035	77	
6	3.59	0.047	trace	0.007	0.054	87	0.5 gram sodium nucleate per os.
7	*3.41	0.029	none	0.010	0.039	74	
8	*3.28	0.056	none	0.010	0.066	85	1.0 gram sodium nucleate per os.
9	3.28	0.029	none	0.009	0.038	76	
10	3.56	0.043	none	0.013	0.056	77	1.0 gram sodium nucleate per os.
11	3.44	0.032	none	0.010	0.042	76	
12	3.78	0.065	0.004	0.011	0.080	81	2.0 gram sodium nucleate per os.
13	3.43	0.029	none	0.012	0.041	71	
14	3.52	0.029	none	0.010	0.039	74	

* Rations not entirely consumed.

The average excretion of purine nitrogen by the feces was: for periods 1-3, 0.029; 4-6, 0.028; 7-10, 0.022; and 11-14, 0.025 gram.

The total purine-allantoin output for these periods is a nearly constant quantity. Expressed in terms of nitrogen, it lies on all but five occasions between the narrow limits of 38 and 42 mgm. and it has an average value over all of 39, or, in twenty-four hours, 19.5 mgm. This is a little more than 1 per cent of the total nitrogen excretion. The endogenous purine metabolism of the monkey

TABLE II.

PERIOD	GRAMS NITROGEN IN 48 HOURS					ALLANTOIN N IN PER CENT OF TO- TAL PURINE- ALLANTOIN N	REMARKS
	Total	Allantoin	Uric acid	Purine bases	Total purine- allan- toin N		
15	3.39		none	0.010			
16	3.50	0.030	none	0.010	0.040	75	
17	3.60	0.027	none	0.011	0.038	71	
18	3.47	0.030	none	0.011	0.041	73	
19	3.64	0.030	none	0.011	0.041	73	
20	3.74	0.031	none	0.011	0.042	74	
21	*3.29	0.030	none	0.010	0.040	75	
22	*3.30	0.028	none	0.011	0.039	72	0.1295 gram allantoin <i>per os</i> .
23	3.44	0.029	none	0.011	0.040	73	
24	3.52	0.042	none	0.010	0.052	81	0.2765 gram allantoin <i>per os</i> .
25	3.51	0.028	none	0.012	0.040	70	
26	3.71	0.050	none	0.012	0.062	81	0.2718 gram allantoin <i>per os</i> .
27	*3.45	0.027	none	0.010	0.037	73	
28	*3.37	0.089	none	0.010	0.099	90	0.1988 gram allantoin subcutaneously.
29	*3.15	0.024	none	0.009	0.033	73	
30	3.41	0.029	none	0.007	0.036	81	
31	*3.28	0.054	none	0.011	0.065	83	0.0946 gram allantoin subcutaneously.
32	*3.15	0.029	none	0.012	0.041	71	
33	*3.50	0.025	none	0.014	0.039	64	
34	*3.42	0.054	none	0.013	0.067	81	0.0963 gram allantoin subcutaneously.
35	*2.89	0.027	none	0.012	0.039	69	
36	*2.69	0.026	none	0.012	0.038	68	Diarrhoea.
37	*3.53	0.035	0.004	0.011	0.050	70	2.0 grams sodium nu- cleate <i>per os</i> .
38	*3.42	0.024	trace	0.012	0.036	67	
39	3.60	0.020	none	0.010	0.030	67	
40	3.75	0.045	0.004	0.013	0.062	73	2.0 grams sodium nu- cleate <i>per os</i> .
41	*3.40	0.025	0.001	0.012	0.038	66	
42	*3.20	†(0.019)	none	0.014			

* Rations not entirely consumed.

† The allantoin determination of this period was lost. The figure given was obtained on period 43.

Average excretion of purine nitrogen by the feces: for periods 15-21, 0.014;
22-36, 0.011; 37-42, 0.011 gram.

is evidently pitched upon a comparatively low level. It happens that we can compare our subject in this respect with a dog of identical weight (4.7 kgm.); this animal, as reported by Hirokawa,²³ on a diet all but free from purines and excreting 1.64 grams of total urinary nitrogen, eliminated daily 0.159 gram of purine + allantoin nitrogen. This is eight times as much as the monkey.

Uric acid could be detected in none of the "normal" urines; if this does not positively prove its absence, it shows at least that any quantity actually eliminated must have been excessively minute.

Purine bases on the other hand are excreted regularly in relatively considerable amount. The nitrogen present in this form varies between 7 and 14 mgm. Each extreme is an exceptional figure; on the whole the purine base excretion exhibits great regularity, seventeen of our twenty-three normal values lying between 10 and 12 mgm. The average of all is 10.5 (daily, 5.25) mgm. of nitrogen. The monkey, it appears, is one of the few animals known to excrete purine bases in greater quantity than uric acid.

For endogenous *allantoin* the lowest nitrogen value recorded is 20 mgm., the highest, 32. Here again a much greater approach to constancy is made than these isolated figures would indicate. There is reason to believe that the minimum (period 39) was reached as an indirect result of previous feeding with sodium nucleate; the two nearest to it are 24 and 25 (periods 29 and 33), and even these are in all probability similarly related to the allantoin injections.²⁴ Be this as it may, there are eighteen periods for which the allantoin nitrogen falls between 27 and 31 mgm. The average of the whole twenty-three is 28. If for the reasons stated we neglect the three lowest, the average is 29. This means a daily excretion of 14.5, the equivalent of 0.041 gram of allantoin.

These perfectly consistent results confirm and are confirmed by the reports of Wiechowski upon what were doubtless other species of monkey. They are entirely in line with the demonstration by Wells of the uricolytic capacity of the *Macacus* liver. They afford no evidence whatever of an approach to the human type. From the point of view of endogenous purine metabolism, the monkey decidedly ranks with the lower mammals. Yet the urin-

²³ Hirokawa: *loc. cit.*

²⁴ See the second last paragraph of this paper.

ary picture presents peculiarities. A reference to the seventh column of Tables I and II shows that the share taken by allantoin in the sum of purine derivatives is not precisely that which is typical of the majority of animals. The figure expressing it—what we may call the “allantoin ratio”—may in “normal” periods be as low as 64 or as high as 81 per cent but it generally remains somewhere between 70 and 78. It never attains the high values recorded for species so divergent as the dog and the pig. On the average only 73 per cent of the total nitrogen of endogenous purine origin takes the form of allantoin; as much as 27 per cent is left for the purine fraction. This state of affairs resembles the condition found by Wiechowski to be characteristic for the horse; here the “allantoin ratio” was in one case 50, in another 79.²⁵ Curiously enough the urine of this animal presents another similarity to the monkey’s in the great preponderance of purine bases over uric acid. According to a few analyses of sheep’s urine made in this laboratory—upon which, however, we do not at present place too much reliance²⁶—the “allantoin ratio” of this animal also is rather low while its purine fraction is about equally divided between the bases and uric acid. A full explanation of these interesting relations is not yet possible.

*The fate of sodium nucleate and allantoin in the organism
of the monkey.*

We have thus far taken for granted that in the purine metabolism of our monkey no other products actually arise than those considered. It seemed to us desirable to test the validity of this assumption. The widely held conviction that *man*, in regard to this very point, offers a notable contrast to the lower mammals, would add a special interest to the outcome of such a test. In pursuance of this idea we attempted, in the first place, to ascertain the fate of sodium nucleate administered by the mouth.

²⁵ Wiechowski: *loc. cit.*, 1909. Wiechowski did not determine the purine base content of his horse urines. If this were as high as Schittenhelm and Bendix (*loc. cit.*) found it in other samples, the allantoin ratio in our sense would be lower still.

²⁶ They were made upon the urine of thyroidectomized sheep, and may therefore represent ratios that are pathological. They will be published later in another connection.

The sodium nucleate used was obtained from Merck. It contained in the air-dried state 13.99 per cent of nitrogen. Its purine content was determined by twice repeated precipitation with copper sulphate and sodium bisulphite after hydrolysis with 3 per cent sulphuric acid; duplicate analyses gave 8.67 and 8.69, average, 8.68 per cent of purine nitrogen.

The quantity administered was so chosen as to be not altogether out of proportion with the extent of the endogenous metabolism. The smallest dose contained rather less purine-ring nitrogen than the combined allantoin and purines of the normal urine and the largest not four times this amount. Bigger ones, it was feared, might by bringing about of themselves an abnormal condition defeat the very object of their administration. Certain experiments of Hirokawa, in which the natural relation between uric acid and allantoin was altered by repeated administration of sodium nucleate,²⁷ afford evidence that this is a consideration to be borne in mind. Indeed our own maximal dose, small as it was in comparison, was not always without perceptible after-effect upon the regular endogenous metabolism.²⁸

In Tables I and II there are recorded seven experiments with sodium nucleate. Inspection of the data for fecal purines makes it almost certain that absorption was in every case complete. An increase of urinary allantoin follows every dose. After the larger ones uric acid also is excreted in measurable amounts. An effect upon the purine bases might, to judge from their share in the endogenous excretion, have reasonably been expected but none is with certainty discernible; a slight rise in this fraction does sometimes (periods 10 and 40) take place but at other times there is a fall and the rise is paralleled on other occasions (for example 33 and 42) quite unconnected with nucleate feeding. The net result upon the "allantoin ratio" is to raise it somewhat above its earlier level. Forty-eight hours generally suffices for the complete elimination of the catabolic products. But on two occasions (38 and 41) the excretion of uric acid undoubtedly stretched into the succeeding period, and one may with probability assume that a portion of the allantoin accompanying it was likewise derived from the exogenous source. This indicates for the monkey, as compared with the dog, a rather slow rate either of absorption or of elimination.

The experiments, as was to be expected, reveal allantoin as an important product of the exogenous as well as the endogenous metabolism of purines. They yield in addition positive evidence of the appearance of uric acid as its precursor. In their quantita-

²⁷ Hirokawa: *loc. cit.*

²⁸ This is discussed in the last paragraph but one of the paper.

tive aspects they are not so satisfactory. In Table III the results are so arranged as to show what fraction of the purine nitrogen fed was actually accounted for.

As the norm from which to estimate the actual increase of allantoin in any given experiment we have taken the mean between the output of the period immediately preceding and that of the earliest subsequent period in which no exogenous excretion is detectable. (The latter, as we have noted, is generally the period immediately after the experimental one but sometimes the next again.) The intake is measured as four-fifths of the total purine nitrogen administered, that fraction only being contained in the purine ring and available for transformation into uric acid or allantoin.

We recover, in ascertained combinations, with small doses about half,²⁰ sometimes as little as an eighth with larger ones, and as the

TABLE III.

PERIOD	PURINE-RING N FED	MOM. OF NITROGEN RECOVERED			PERCENTAGE RECOVERY
		As uric acid	As allantoin	Total	
4	35	0	16	16	46
6	35	trace	19	19	54
8	70	0	27	27	39
10	70	0	12	12	17
12	140	4	35	39	28
37	140	4	13	17	12
40	140	5	30	35	25

average of all only 32 per cent. In face of the almost certainly complete absorption of the nucleate, how is the deficit to be explained? Perhaps the first possibility that suggests itself is that allantoin may be no end product, but liable itself to catabolic changes. It is true that metabolic processes do not often exhibit the irregularity that would then seem to characterize the destruction of allantoin. Nevertheless we felt bound to submit the possibility to the test of experiment. On three occasions allantoin was administered by mouth, on three others, by subcutaneous injection. The same principles as before were applied to the selection of doses

²⁰ In an earlier report already referred to (Hunter and Givens: *loc. cit.*, 1912) we alleged a recovery, after the first dose, of 90 per cent; this statement involved, as we have since discovered, a gross error in calculation.

and to the interpretation of the data obtained. The results are brought together in Table IV.

Between the two sets of allantoin experiments with their almost directly contradictory results we have no hesitation in selecting the injections as the decisive ones. In these alone are we absolutely certain that the substance entered unaltered into the organism; and it is the fate of allantoin within the organism, and that only, that concerns us. It has been objected to the parenteral modes of administration that they may induce disturbances of general metabolism so profound as to obscure the true interpretation of results.³⁰ The objection is doubtless in many cases sufficiently well grounded, but in the present one no greater disturbance was noticed than might have been produced by the injection of so

TABLE IV.

PERIOD	METHOD	MILLIGRAMS ALLANTOIN N		PERCENTAGE RECOVERY
		Administered	Recovered	
22	oral	45.8	0	0
24	oral	98.0	13	13
26	oral	96.3	22	22
28	subcutaneous	70.4	63	90
31	subcutaneous	33.5	25	75
34	subcutaneous	34.1	28	82

much water. The feeding experiments on the other hand are attended by numerous uncertainties. The warmth and alkaline reaction of the intestinal contents are of themselves likely, according to Wiechowski,³¹ to bring about the rapid destruction of allantoin. Add to this the slight solubility of the substance and the possible action of bacteria, and it would not be at all surprising if none of the first small dose we gave had ever reached the blood stream. It is to such factors that we incline at present to attribute the poor recovery of ingested allantoin.

However this may be, the results of the injection experiments are perfectly definite. Small doses of allantoin, subcutaneously introduced, are recoverable from the urine of the next forty-eight

³⁰ Cf. Schittenhelm: *loc. cit.*, 1909.

³¹ Wiechowski: *loc. cit.*, 1910.

hours almost as completely as if they had been directly dissolved therein. It seems impossible to escape from the conclusion that, if the monkey tissues possess any capacity at all for the destruction of allantoin, it is so poorly developed as to have but little practical significance.

The results of our allantoin experiments with the monkey are paralleled by those reported upon other species. After ingestion there have been recovered widely varying, often very low, proportions: in the dog, 70 (Minkowski³²), 90 (Poduschka³³) and 31 per cent (Levene and Medigreceanu³⁴); in the pig, nearly 100 (Schittenhelm³⁵); in man, 17 (Minkowski³⁶), 30-50 (Poduschka³⁷), 34 (Wiechowski³⁸) and 30-38 per cent (Schittenhelm and Wiener³⁹). Subcutaneous administration has been seldom employed: once by Schittenhelm⁴⁰ on the pig with quantitative recovery and thrice by Wiechowski⁴¹ on man with yields up to 88 per cent.⁴²

Most writers are in fact at one in regarding allantoin as a terminal and not merely an intermediary product of metabolism. Our contribution to the question serves but to emphasize the correctness of this view. To explain the deficit of our sodium nucleate experiments we must look then to other factors than allantoin destruction. What these actually are, it is as yet impossible to say. But there are certain considerations, which appear to us to suggest the direction in which they are to be sought.

There is among recent investigators a pretty general agreement that, when nucleic acid is fed to an animal like the dog, its constituent purines can be quantitatively accounted for (or nearly so) by the excess of allantoin, uric acid and bases eliminated during the

³² Minkowski: *Arch. f. exp. Path.*, xli, p. 375, 1898.

³³ Poduschka: *loc. cit.*

³⁴ Levene and Medigreceanu: *loc. cit.*

³⁵ Schittenhelm: *loc. cit.*, 1910.

³⁶ Minkowski: *loc. cit.*

³⁷ Poduschka: *loc. cit.*

³⁸ Wiechowski: *Arch. f. exp. Path.*, lx, p. 185, 1909.

³⁹ Schittenhelm and Wiener: *loc. cit.*

⁴⁰ Schittenhelm: *loc. cit.*, 1910.

⁴¹ Wiechowski: *Arch. f. exp. Path.*, lx, p. 185, 1909.

⁴² Schittenhelm and Seisser (*Zeitschr. f. exp. Path.*, vii, p. 116, 1909) injected allantoin intravenously into the rabbit. Only an abstract of their paper is available (*Biochem. Zentralbl.*, ix, p. 746) and the quantitative details are not there given. The above list includes, we believe, all other experiments in which the recovery was quantitatively determined.

next twenty-four hours. Schittenhelm,⁴³ for example, reports recoveries of 88-102, Hirokawa,⁴⁴ of 72-88 per cent. We may therefore take it as demonstrated that in the dog no other products of purine catabolism need be looked for. It appears at least equally well established that man, after the ingestion and undoubted absorption of nucleate of sodium, excretes as uric acid only a fraction (according to the particularly careful experiments of Frank and Schittenhelm⁴⁵ from 5 to 41 per cent) of the purine intake; while no perceptible increase of the insignificant allantoin output assists in making good the deficit.⁴⁶

A vehement controversy centers round the explanation of this difference in behavior. According to what is probably at present the prevailing view,⁴⁷ uric acid is in man, as much as in the lower animals, an intermediary product only; and its destruction is accomplished in his case by a peculiar type of uricolysis leading not to or even through allantoin, but, by steps not yet elucidated, to urea. Another school of opinion denies to human tissues every kind of uricolytic power; regards uric acid therefore as a terminal product; and attributes the observed deficit after nucleate feeding to destruction of the purine ring before absorption.⁴⁸

We do not propose to enter here into the merits of this controversy. What is of interest to us is that in response to the enteral administration of sodium nucleate the monkey reacted, not like the animal with which its high allantoin excretion would associate it, but like man. It is hardly likely that we are dealing here with two unrelated processes leading to the same exceptional result. It may be assumed with greater probability that that theory of the fate of ingested nucleic acid which is ultimately found to hold good for the human animal will equally fit the monkey. If the experimental facts require us to postulate for the former a particular kind of uricolytic enzyme, that enzyme is probably active in the latter also. We should be led, then, to the unexpected con-

⁴³ Schittenhelm: *loc. cit.*, 1909.

⁴⁴ Hirokawa: *loc. cit.*

⁴⁵ Frank and Schittenhelm: *Zeitschr. f. physiol. Chem.*, lxiii, p. 269, 1909.

⁴⁶ Schittenhelm and Wiener: *loc. cit.*

⁴⁷ Cf. Brugsch and Schittenhelm: *Der Nucleinstoffwechsel*, Jena, 1910, pp. 41-45, where the literature of the controversy will be found.

⁴⁸ See the papers of Wiechowski already cited; also Siven: *Arch. f. d. ges. Physiol.*, cxlv, p. 283, 1912.

clusion that the monkey disposes of intermediary purines by two independent mechanisms: the one peculiar to the lower mammals and leading to allantoin, the other ending in urea and encountered in no other species save man. This, if true, would be a striking illustration of the monkey's philogenetic relations, but it hardly bears the stamp of probability. It is easier to believe that the undeniable destruction of purine nuclei, which we observed, was really accomplished in the monkey's intestine before absorption had taken place. If this happens in the monkey's intestine, it may also happen in man's; and while our experiments have no direct bearing on the problems of human metabolism, we think that they afford some indirect evidence against the assumption of an extensive uricolysis in the human organism.

One curious circumstance deserves perhaps more particular notice than we have yet bestowed upon it. If the data of Table I be reviewed, it will be observed that in no case there recorded does the administration of sodium nucleate produce any effect other than the immediate one of an increase in the total purine metabolism.⁴⁹ There is after every dose a prompt return to the same "normal" level of allantoin excretion. It is otherwise with the experiments of Table II. Here may be noted from period 27 onwards an almost uninterrupted sinking of the "normal" figures for allantoin. Each rise of the output, whether resulting from allantoin injection or from nucleate feeding, is followed, more or less immediately, by a fall below the previous standard. Most striking are the drop from 27 to 24 mgm. after the first allantoin injection, from 29 to 25 after the second and from 26 to 20 after the first dose of nucleate. The summation of these effects brings the allantoin nitrogen finally down to two-thirds of its original value. This phenomenon, in relation at least to nucleate feeding, has already been noticed by Schittenhelm in experiments upon the dog and the pig.⁵⁰ Our observation, that it may follow simple allantoin injections, suggests that in both cases it is the temporary excess of cir-

⁴⁹ It may be worth recording that we determined regularly during this series the excretion of ammonia and creatinine and that these exhibited no single departure from a very narrow range of values; the ammonia nitrogen varied between 0.039 and 0.049 gram, creatinine nitrogen between 0.123 and 0.134, nucleate administration having no perceptible effect upon either.

⁵⁰ Schittenhelm: *loc. cit.*, 1909 and 1910.

culating allantoin that in some way is responsible. But the same remarkable after-effect has been seen to follow the administration of histidine hydrochloride, which has no relation at all to the production of allantoin.⁵¹ The exact significance of the lowered output remains obscure. It was not accompanied in the monkey by any uniform variation of the purine base excretion.

SUMMARY.

In the urine of the guenon monkey (*Cercopithecus*) allantoin accounts for about 73 per cent of the nitrogen arising from the catabolism of endogenous purines. The remainder appears principally as purine bases, uric acid being practically absent when the diet is purine-free. The allantoin is a true end product. When purines are ingested in the form of sodium nucleate, the allantoin output is increased and uric acid may appear as an intermediate product. Only 12 to 54 per cent of the total purine intake is thus accounted for. The deficit is probably the result of decomposition prior to absorption. There is no evidence in this monkey of an approach to the human type of nuclein metabolism.

(Experiments upon the fate of individual purines in the monkey organism are now in progress, and will be reported upon in a later communication.)

⁵¹ See Abderhalden and Einbeck: *Zeitschr. f. physiol. Chem.*, lxi, p. 322, 1909; and Abderhalden, Einbeck and Schmid: *ibid.*, lxxviii, p. 395, 1910.

ABSORPTION FROM THE STOMACH—A REPLY TO LONDON.

BY OTTO FOLIN AND HENRY LYMAN.

(From the Biochemical Laboratory of the Harvard Medical School.)

(Received for publication, November 12, 1912.)

In our communication on Absorption from the Stomach¹ we gave figures which in our judgment leave no room for doubt concerning the absorption of protein digestion products from the stomach. To E. S. London our results are evidently not at all convincing and in a recent number of the *Zeitschrift für physiologische Chemie*² he has endeavored to make clear several reasons why our results are inconclusive. In view of the great amount of interesting experimental work done by London and his associates on the subject of absorption (by methods entirely different from ours) it is not at all strange that he should be unable to accept our conclusion, diametrically opposite as it is to the conclusion reached by himself.

The three main points raised by London against our work are briefly discussed below.

1. The increase in the non-protein nitrogen of the blood during our absorption experiments might be due to absorption from the intestinal tract of our fasting animals.

This is to us an interesting argument in view of the fact that it heretofore has been practically impossible to demonstrate any increase in the non-protein nitrogen of the blood as a result even of carefully planned absorption experiments with the intestine. As a matter of fact the absorption which we obtained from the stomach is more rapid than any absorption from the small intestine which we have observed as a result of ordinary feeding

¹ This *Journal*, xii, p. 259.

² *Zeitschr. f. physiol. Chem.*, lxxxi, p. 283, 1912.

Numerous experiments made in this laboratory have moreover shown that unless there is in the intestine a fair amount of material suitable for absorption, the non-protein nitrogen of the blood remains just about stationary during the short periods covered by our experiments. Some such experiments made for other purposes have already been recorded. See, for example, the two creatinine experiments³ and the experiment on the absorption of tyrosine,⁴ in all of which virtually no increase in the non-protein nitrogen of the blood was obtained.

We would further call attention to the fact that urea is particularly rapidly absorbed from the stomach.⁵ London will have to concede that the accumulation of urea in the blood accompanying the disappearance of urea from the stomach can be due only to the absorption of urea from the stomach. And having conceded this, there is, so far as we can see, no reason why the absorption of glycocoll, etc., should be less readily conceded.

2. To London it seems strange that we should affirm that something was absorbed from Witte's peptone in the stomach, when we obtained only 40 mgm. of non-protein nitrogen per 100 cc. of blood, yet find no absorption of creatinine, although in one creatinine experiment the corresponding figure for the non-protein nitrogen of the blood was 53 mgm.

The amount of the initial non-protein nitrogen in the blood is determined by the previous condition of the animal. It is only the increase during the absorption experiment which proves anything. In the creatinine experiment referred to⁶ the non-protein nitrogen rose from 52 to 52 mgm., i.e., nothing in ninety minutes. In the peptone experiment on the other hand the corresponding figure rose from 34 to 40 during the same length of time.

3. London finally falls back on the vague and all too common argument that the facts observed have anyhow no bearing on what happens normally. Because dogs die in the course of a few days when the pylorus is closed by means of a ligature, London would insist that the absorption from the stomach of cats obtained in the course of a few minutes after the application of such a ligature

³ This *Journal*, xii, p. 262-3.

⁴ *Ibid.*, xii, p. 147-8.

⁵ *Ibid.*, xii, p. 263.

⁶ *Ibid.*, xii, p. 263.

is to be explained as a pathological rather than as a physiological phenomenon. We are disposed not to argue over this point but would only remark that a similar view can then be advanced against almost every experiment with animals, to say nothing of experiments of every description with isolated organs.

It should be noted that London's results stand in a measure alone. Tabler,⁷ Salaskin,⁸ Cohnheim,⁹ Schewnert,¹⁰ and Lang¹¹ all have reached the conclusion that nitrogenous products disappear from the stomach through direct absorption. Taken in conjunction with the results of all these investigators ours might be regarded as only a verification (by a more direct method) which at the same time proves that the nitrogenous products absorbed from the stomach reach the circulation in non-protein form.

Finally we wish to thank London for his courtesy in sending us an advance copy of his note on our work and to express our regret that he just now (as he has privately informed us) will be unable to publish further experimental work on the problem. As we indicated in our original paper¹² London's experimental results are not necessarily inconsistent with ours. His absorptions may have been hidden by excessive secretions into the stomach of various kinds of nitrogenous materials, while in our experiments (which anyhow were of relatively short duration) such secretion could not hide the accumulations in the blood.

⁷ *Zeitschr. f. physiol. Chem.*, xlv, p. 185, 1905.

⁸ *Ibid.*, lii, p. 167, 1907.

⁹ *Ibid.*, lviii, p. 64, 1908.

¹⁰ *Ibid.*, li, p. 535, 1907.

¹¹ *Biochem. Zeitschr.*, ii, p. 225, 1906.

¹² *This Journal*, xii, p. 264.



ANNOUNCEMENT.

The Directors of the *Journal of Biological Chemistry* take pleasure in announcing that beginning with the February issue of 1913 (Volume XIV, No. 1) the subscription price of the *Journal* to domestic subscribers will be reduced from \$4.00 to \$3.00 per volume; to foreign subscribers, \$3.25.

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A. N. RICHARDS,
Secretary.



ON THE INTENSITY OF URINARY ACIDITY IN NORMAL AND PATHOLOGICAL CONDITIONS.

By LAWRENCE J. HENDERSON AND WALTER W. PALMER.¹

(From the Chemical Laboratory, Massachusetts General Hospital.)

(Received for publication, November 2, 1912.)

The researches of v. Röhrer, Höber and one of us² have shown that both normally and pathologically the true acidity (concentration of ionized hydrogen) in human urine is subject to considerable inconstancy. On the whole the variation is such as is characteristic of a solution containing phosphoric acid and base in which the latter ranges from that amount required to form monosodium phosphate to nearly that amount required to form disodium phosphate; and such fluctuation in the urinary phosphates is in fact the principal cause of the varying acidity, both "actual" and "potential."

This definite information might well suffice in the circumstances were it not for the fact that the intensity of urinary acidity is, among several, the most immediate indication of the magnitude of a process which constitutes one of the fundamental regulating activities of the organism: the carefully balanced excretion of acid whereby the neutrality of the body is preserved.³ Accordingly precise and numerous data of urinary acidity, both normally and pathologically, appear to be desirable. Fortunately the researches of Sørensen make this task an easy one. His researches have made possible the convenient use of a considerable variety of indicators⁴ and provided ingenious and convenient refinements

¹ Henry P. Walcott Fellow in Clinical Medicine, Harvard Medical School.

² *Pflüger's Archiv*, lxxxvi, p. 586, 1901; *Beitr. z. chem. Physiol.*, iii, p. 525, 1903; *Biochem. Zeitschr.*, xxiv, p. 40, 1910.

³ L. J. Henderson: this *Journal*, ix, p. 403, 1911.

⁴ See numerous papers in *Comptes rendus des travaux du Laboratoire de Carlsberg*.

which enhance their accuracy. Our investigations, recorded in this paper, yield a confirmation, if such be necessary, of the reliability of these methods.

We have as before estimated the urinary reaction after the addition of indicators by matching colors of urinary samples with those of standard solutions of known reaction. The standard solutions were made up as follows:

TABLE I.

NO.	NaH_2PO_4	Na_2HPO_4	$\frac{+}{\text{H}}$	INDICATOR	
1		0.1000N	9.27	} Phenolphthalein.	} Sodium alizarine sulphonate.
2	0.0001N	0.0480N	8.7		
3	0.0001N	0.0120N	8.0		
4	0.0166N	0.0833N	7.48	} Neutral red.	
5	0.0010N	0.0060N	7.38		
6	0.0010N	0.0023N	6.90		
	CH_3COOH	CH_3COONa			
7	0.0009N	0.0920N	6.70	} <i>p</i> -Nitrophenol.	
8	0.0023N	0.0920N	6.30		
9	0.0046N	0.0920N	6.00		
10	0.0092N	0.0920N	5.70		
11	0.0230N	0.0920N	5.30		
12	0.0460N	0.0920N	4.90	} Methyl red.	
13	0.0920N	0.0920N	4.70		

In recording hydrogen ion concentration it appears to be both rational and convenient in the present case to use logarithmic notation as employed by Sørensen and others, rather than to record the actual concentrations, because the significant variation is in the logarithm of the numbers which represent the quantity $\frac{+}{H}$. All such logarithms are of course negative and for convenience the minus sign is omitted. The following table presents the conversion of the logarithmic notation into actual concentrations of ionized hydrogen.

Our first care was to make a more detailed study of the effect of dilution of the urine upon its reaction and to see if a variety of indicators yielded concordant results. It soon appeared that the reaction of undiluted urine could be readily estimated by employing colored standard solutions (Sørensen's method). The varying

TABLE II.

LOG.	$\frac{+}{H}$	LOG.	$\frac{+}{H}$
4.6	250×10^{-7}	6.4	4.0×10^{-7}
4.8	160×10^{-7}	6.6	2.5×10^{-7}
5.0	100×10^{-7}	6.8	1.6×10^{-7}
5.2	63×10^{-7}	7.0	1.0×10^{-7}
5.4	40×10^{-7}	7.2	0.63×10^{-7}
5.6	25×10^{-7}	7.4	0.40×10^{-7}
5.8	16×10^{-7}	7.6	0.25×10^{-7}
6.0	10×10^{-7}	7.8	0.16×10^{-7}
6.2	6.3×10^{-7}	8.0	0.10×10^{-7}

colors of urine are as a rule easily reproduced in standard solutions with all desirable accuracy by the addition of *p*-nitrophenol, methyl orange, alizarine sulphonate or bismark brown. Thereafter the substance which is to be used as indicator may be added in equal concentrations to urine and colored standard solutions, and that one of the latter which still corresponds to the urine in color may be selected.

After the reaction of urine has been estimated in this manner with whatever may be convenient, it is an easy task to determine the effect of dilutions upon the reaction by diluting both urine and standard solution equally. The following table presents a fair sample of our data of the reaction of diluted and undiluted urine.

TABLE III.

INDICATOR	REACTION		INDICATOR	REACTION	
	Undiluted	Diluted		Undiluted	Diluted
Para-nitrophe- nol.....	5.40	5.40	Sodium alizarine sulphonate....	5.00	5.00
	6.70	6.70		5.00	5.00
	5.52	5.52		6.82	6.82
	5.82	5.82		7.10	7.10
	5.00	5.00		7.22	7.22
Methyl red....	5.00	5.22	Methyl red.....	7.10	7.10
	4.82	5.00		6.82	6.82
	5.12	5.30		7.10	7.10
	4.70	4.82		7.22	7.22
				7.10	7.10

Evidently therefore, except in the easily explained case of very acid urines, the effect of dilution is unimportant and upon the whole dilution has the same effect upon the reaction of urines and of standard solutions, as must indeed be the case for similar "balanced solutions." This conclusion confirms previous work and gives convenient latitude in the choice of further procedure.

Observations upon a variety of indicators were no less satisfactory. Those which we have employed include *p*-nitrophenol, methyl orange, methyl red, neutral red, alizarine sulphionate and phenolphthalein. Except the last, each of these may be compared in certain ranges of reaction with at least one other and in all cases there is very satisfactory accord between the results with different indicators. The following sample of our numerous data illustrates the nature of the case.

TABLE IV.

NUMBER	PARA-NITROPHENOL, UNCOLORED STANDARDS, DILUTED	SODIUM ALIZARINE SULPHONATE, UNCOLORED STANDARDS, DILUTED	PARA-NITROPHENOL, COLORED STANDARDS, DILUTED	SODIUM ALIZARINE SULPHONATE, COLORED STANDARDS, DILUTED	PARA-NITROPHENOL, COLORED STANDARDS, UN-DILUTED	SODIUM ALIZARINE SULPHONATE, COLORED STANDARDS, UN-DILUTED
1	5.40	5.40	5.40	5.40	5.40	5.40
2	5.00	5.00	5.00	5.00		
3	5.82	5.82	5.82	5.82	(?)	5.82
4	6.70	6.52	6.70	(?)	6.70	6.52
5	5.52	5.52	5.52	5.52	5.52	(?)
6	5.40	5.30	5.40	5.22		
7	5.30	5.30	5.30	5.30		
8	5.52	5.52	5.52	5.52		
9	5.82	5.82	5.82	5.82	5.82	5.82
10	6.40	6.40	6.70	6.70		
11	5.52	5.82	5.82	5.82		
12	5.40	5.40	5.52	5.40		

TABLE V.

NUMBER	PARA-NITROPHENOL, UNCOLORED STANDARDS, DILUTED	SODIUM ALKALINE SULPHONATE, UNCOLORED STANDARDS, DILUTED	METHYL RED, UNCOLORED STANDARDS, DILUTED	PARA-NITROPHENOL, COLORED STANDARDS, DILUTED	SODIUM ALKALINE SULPHONATE, COLORED STANDARDS, DILUTED	METHYL RED, COLORED STANDARDS, DILUTED
1	5.00	5.00	5.00	5.00	5.00	5.00
2	5.18(?)	4.82	5.00	5.00	5.00	5.00
3	5.22	5.22	5.22	5.22	5.22	5.22
4	5.00	5.00	5.12	5.12	5.12	5.12
5	5.18	5.00	5.30	5.18	5.00	5.30
6	5.18	5.18	5.30	5.18	5.18	5.30
7	5.18	5.18	5.30	5.18	5.18	5.30
8	5.18	5.18	5.30	5.18	5.18	5.30
9	5.00	4.82	5.18	5.00	4.82	5.18
10	5.30	5.22	5.30	5.30	5.22	5.30

TABLE VI.

NUMBER	NEUTRAL RED, UNCOLORED STANDARDS, DILUTED	SODIUM ALKALINE SULPHONATE, UNCOLORED STANDARDS, DILUTED	NEUTRAL RED, UNCOLORED STANDARDS, DILUTED	SODIUM ALKALINE SULPHONATE, COLORED STANDARDS, DILUTED	NEUTRAL RED, COLORED STANDARDS, UNDILUTED	SODIUM ALKALINE SULPHONATE, COLORED STANDARDS, UNDILUTED
1	6.82	6.82	6.82	6.82	6.82	6.82
2	7.10	7.10	6.82	7.10	6.82	7.10
3	7.22	7.22	7.22	7.22	7.22	7.22
4	7.00	7.00	7.00	6.82	7.00	(?)
5	7.10	7.10	7.10	7.10	7.10	7.10
6	7.22	7.22	7.22	7.22	7.22	7.22
7	7.10	6.82	7.10	7.10	7.10	(?)
8	7.10	7.00	7.10	7.10	7.10	(?)
9	7.45	7.10	7.45	7.10	7.10	(?)
10	7.45	7.40	7.45	7.40	7.45	7.12

TABLE VII.

NUMBER	SODIUM ALIZARINE SULPHONATE UN- COLORED STAND- ARDS, DILUTED	METHYL RED, UN- COLORED STAND- ARDS, DILUTED	SODIUM ALIZARINE SULPHONATE, COLORED STAND- ARDS, DILUTED	METHYL RED, COL- ORED STAND- ARDS, DILUTED	SODIUM ALIZARINE SULPHONATE, COLORED STAND- ARDS, UN-DILUTED	METHYL RED, COL- ORED STAND- ARDS, UN-DILUTED
1	5.00	5.00	5.00	5.00	5.00	5.00
2	5.22	5.22	5.22	5.22		5.22
3	5.00	5.12	5.12	5.12	5.00	5.00
4	4.82	5.00	4.82	5.00		4.82
5	5.12	5.22	5.12	5.30		5.12
6	5.00	5.12	5.05	5.12		5.05
7	5.00	5.22	5.00	5.22		5.12
8	5.30	5.30	5.22	5.40		5.22
9	5.30	5.30	5.30	5.30		5.22
10	5.22	5.22	5.22	5.22		5.22
11	4.70	4.82	4.70	4.82		4.70
12	4.82	5.12	4.82	5.12		5.12

These results not only justify the use of any one of several indicators⁴ but also give added assurance of the reliability of the measurements. Even more important however is the fact that the observations are at any time subject to further experimental investigations, because the standard solutions can at any time be reproduced. It is also to be noted that for all practical purposes the reference of urinary reaction to such a series of solutions is quite as satisfactory as reference to the actual hydrogen ion concentration.

Having in this manner confirmed the earlier work and found that the possible choice of indicators is a large one, we have after much experimentation decided upon the following procedure as on the whole a convenient method for actual use.

A number of 250 cc. flasks of good glass are selected. They must be indistinguishable in color and form. In each of ten of these flasks is placed a 10 cc. sample of one of the standard solutions, the volumes are made up to 250 cc. with distilled water and an aqueous solution of alizarine sulphionate

⁴ Certain difficulties were found with alizarine sulphionate in albuminous urine. Methyl red and *p*-nitrophenol, however, are little affected by the presence of albumen and so may be depended upon in these cases. In the range 0.3×10^{-7} — 2.0×10^{-7} alizarine sulphionate changes color so gradually that accurate estimation could not be made. Neutral red, however, is very satisfactory in this range. Similarly *p*-nitrophenol is found unsatisfactory in the range of 50×10^{-7} and 200×10^{-7} .

of sodium is added until the concentration of the indicator is about 0.0003 per cent and exactly equal in all cases. 10 cc. of urine are next introduced into another flask and distilled water and indicator are added. The color of the diluted urine solution is next matched with one of the standard series.

In case the reaction as thus measured is more acid than 5.3 ($(H) = 50 \times 10^{-7}$) further tests are made with methyl red; for the range 5.3 — 6.7 ($50 \times 10^{-7} - 2 \times 10^{-7}$), with *p*-nitrophenol and with neutral red; and for more alkaline urines, which thus far have been encountered only after the administration of alkali, with phenolphthalein.

A. $(H) > 50 \times 10^{-7}$. 10 cc. portions of standard solutions are introduced into carefully selected colorless test tubes (measuring in our experiments 15.5 cm. \times 1.7 cm.) and 10 cc. of urine is introduced into another tube. The standard solutions are each colored with one of the pigments above mentioned to match the color of the sample of urine. In case the pigment is itself an indicator within this range the necessary amount will vary with the reaction of the standard solution.

To standard solutions and urine is then added 0.15 cc. of a saturated solution in 50 per cent alcohol of methyl red, and the color is matched.

B. $50 \times 10^{-7} (H) > 2 \times 10^{-7}$. The estimation is carried out in flasks as with alizarine sulphonate, using, however, *p*-nitrophenol, 0.08 per cent.

C. $2 \times 10^{-7} (H) > 0.5 \times 10^{-7}$. The estimation is made as with alizarine sulphonate but employing neutral red, 0.0006 per cent.

D. $(H) < 0.5 \times 10^{-7}$. Undiluted urine is matched in test tubes against undiluted standard solutions using phenolphthalein as indicator (without previous coloration of standard solutions).

In all cases estimations are made in duplicate.

The results thus far obtained are tabulated below.

TABLE VIII.

Normal cases.

5.30	5.00	5.82	6.10	5.82	7.22	5.70	6.39
6.52	5.70	5.12	7.00	7.22	7.46	5.52	5.00
6.30	5.70	6.30	6.52	7.22	5.70	5.12	4.82
5.82	6.88	5.52	7.46	6.00	6.52	5.52	6.00
5.82	5.40	5.12	5.30	6.40	6.30	7.45	7.46
5.52	7.22	5.45	5.70	6.00	5.70	5.82	5.52
5.70	7.46	5.82	6.52	5.12	5.70	5.30	5.70
5.40	5.70	5.40	5.52	5.70	6.00	5.40	5.30
6.82	5.40	5.12	6.16	5.70	6.00	5.82	5.82
7.22	6.39	5.30	5.52	6.15	7.00	5.82	
6.00	6.39	5.52	6.70	6.70	5.40	7.00	
6.52	5.70	5.40	5.12	7.00	6.52	5.70	
5.82	6.70	5.82	6.52	6.40	5.70	7.10	

Average, 6.03.

Intensity of Urinary Acidity

TABLE IX.

Chronic glomerulo-nephritis, hypertrophy and dilatation of heart with cardiac decompensation.

5.00	5.12	6.30	5.00	5.00	4.82
5.12	6.82	5.00	5.40	5.70	5.30

Average, 5.38.

TABLE X.

Chronic glomerulo-nephritis, hypertrophy and dilatation of heart without cardiac decompensation.

4.52	5.12	5.12	5.30	5.40	5.52	5.40
7.40	7.22	5.00	5.82	7.48	5.12	5.12

Average, 5.70.

TABLE XI.

Acute nephritis.

5.30	4.70	5.12	5.00	5.00	5.30	5.30	5.00
------	------	------	------	------	------	------	------

Average, 5.09.

TABLE XII.

Cardiac decompensation without kidney involvement.

5.22	5.30	5.00	5.52	5.12	5.30	5.70	7.48
5.00	7.48	5.40	5.30	5.30	6.52	5.40	5.30
5.52	6.10						

Average, 5.66.

TABLE XIII.

Lobar pneumonia.

7.10	7.48	5.52	7.10	5.82
6.30	5.40	5.12	6.52	5.30

Average, 6.17.

TABLE XIV.

Pleurisy with effusion (tuberculous).

5.00	5.52	5.00	5.52
5.40	5.30	5.22	5.70
5.52			

Average, 5.35.

TABLE XV.

Acute endocarditis.

5.12	5.00	6.90	5.30	5.82
------	------	------	------	------

Average, 5.63.

TABLE XVI.

Diabetes mellitus (not receiving alkali).

7.10	7.22	7.10	5.30
------	------	------	------

Average, 6.65.

TABLE XVII.

Peptic ulcer.

5.40	7.48	7.22	5.40	4.70
------	------	------	------	------

Average, 6.04.

TABLE XVIII.

Syphilis (all cases with secondary symptoms).

6.70	5.90	6.30	7.22	6.70
------	------	------	------	------

Average, 6.56.

TABLE XIX.

Appendicitis with operation (acute and chronic, without peritonitis).

5.70	6.30	5.70	5.40	5.30	5.82	5.12
6.90	5.70	7.22	5.40	4.70	5.52	5.52
5.82	5.12	4.70	5.40	5.90	5.12	

Average, 5.62.

TABLE XX.

Carcinoma.

Rectum.....	6.70	Stomach.....	7.10
Large intestine.....	7.22	Cheek.....	7.00
Large intestine.....	6.40	Gall bladder.....	5.70
Stomach.....	6.40	Rectum.....	5.30
Breast.....	5.30	Breast.....	5.30
Stomach.....	5.52	Penis.....	5.40
Rectum.....	5.52	Intestine.....	5.82
Intestine.....	5.82	Stomach.....	5.52

Average, 6.00.

Intensity of Urinary Acidity

TABLE XXI.

(1) *Pernicious anaemia.*

4.82	5.12	5.12	5.70	5.30	5.82
5.40	7.10	5.30	5.30	5.00	

Average, 5.45.

(2) *Aplastic anaemia.*

5.82	7.22
------	------

Average, 6.52.

(3) *Secondary anaemia.*

Cause (?).....	5.30	Cause (?).....	5.12
Hemorrhoids.....	5.30	Cause (?).....	4.82
Hemophilia.....	6.30		

Average, 5.37.

TABLE XXII.

Sepsis.

Abscess of thigh.....	5.23	Septic scalp.....	7.52
Empyema.....	5.23	Osteomyelitis.....	6.40
Septic throat.....	5.12	Septic burns.....	5.70
Peritonitis.....	4.82	Tonsillitis.....	6.30
Septic burns.....	5.40	Osteomyelitis of fibula.....	4.82
Septic burns.....	5.30	Osteomyelitis.....	6.00
Peritonitis.....	5.40	Peritonsillar abscess.....	5.12
Alveolar abscess.....	5.52		

Average, 5.25.

TABLE XXIII.

Skin diseases.

Herpes zoster.....	7.10	Eczema.....	7.40
Epithelioma.....	5.40	Psoriasis.....	5.90
Dermatitis venenata.....	6.82		

TABLE XXIV.

Genito-urinary diseases.

Pyuria (cause?).....	5.52	Pyuria (diag. ?).....	5.82
Renal calculus.....	7.12	Renal calculus.....	5.12
Renal calculus.....	4.70	Pyuria (diag. ?).....	6.82
Tubercular kidney.....	6.70	Double colon infection.....	5.40
Pyelo-nephrosis.....	5.70	Tubercular kidney.....	7.40
Perinephritic abscess.....	5.40	Pyelitis.....	5.52
Hypernephroma.....	4.70	Hypernephroma.....	5.40
Tubercular epididymitis.....	5.40	Tubercular epididymitis.....	5.82

TABLE XXV.

Arteriosclerosis with weak heart.

5.72	5.12	5.30	5.40	5.30
------	------	------	------	------

Average, 5.37.

TABLE XXVI.

Typhoid fever.

6.70	5.40	5.00	5.40	6.00	5.00	7.48
------	------	------	------	------	------	------

Average, 5.85.

TABLE XXVII.

Miscellaneous cases.

Chorea minor.....	5.82	Taenia saginata.....	5.30
Hyperacidity.....	5.40	Taenia saginata.....	5.30
Brain tumor.....	6.10	Tabes dorsalis.....	6.82
Diabetes insipidus.....	5.12	Unexplained fever.....	5.52
Addison's disease.....	7.45	Chronic arthritis.....	5.12
Unexplained temperature.....	5.52	Chronic arthritis.....	5.20
Unexplained temperature.....	5.52	Unexplained fever.....	7.48
Unexplained high blood pressure.....	5.52	Tuberculous meningitis.....	5.30
Tuberculous peritonitis.....	5.40	Dilatation of arch of aorta.....	7.00
Elephantiasis.....	6.82	Neurasthenia.....	5.70
Unexplained vomiting.....	5.52	Acute rheumatic fever with chorea and aortic regurgitation.....	7.48
Trichinosis.....	6.10	Trichinosis.....	6.00
Chronic bronchitis.....	7.45	Phlebitis.....	5.00
Chronic bronchitis.....	6.52	Hyperacidity.....	6.52
Acute tonsillitis.....	7.00	Sciatica (cause?).....	5.00
Acute tonsillitis.....	5.70	Pyloric stenosis (cause ?).....	7.40
Cholelithiasis.....	5.82	Chronic rheumatism.....	5.52
Brain tumor.....	5.12	Acute rheumatic fever.....	6.40
Unexplained vomiting.....	6.30	Anaemia with enlarged spleen.....	5.52
Abdominal tumor.....	5.52	Bothriocephalus latus.....	5.82
Abdominal tumor.....	7.45	Chronic rheumatism.....	6.70
Hyperacidity.....	7.45	Persistent vomiting of pregnancy.....	7.48
Lumbar caries with abscess.....	6.00	Persistent vomiting of pregnancy.....	7.00
Tabes dorsalis.....	5.30	Persistent vomiting of pregnancy.....	5.70
Acute rheumatic fever.....	5.30	Persistent vomiting of pregnancy.....	5.40
Plumbism.....	7.12	Pulmonary tuberculosis.....	6.10
Myelogenous leukemia.....	5.30	Pulmonary tuberculosis with pneumothorax.....	5.52
Multiple sclerosis.....	6.70	Chronic rheumatism.....	7.10
Malaria.....	5.40		
Hodgkin's disease.....	7.40		
Gastric neurosis.....	6.52		
Plumbism.....	7.48		
Trichinosis.....	4.70		
Unexplained temperature.....	5.12		

TABLE XXVIII.

Summary of averages arranged in order of highest acidities.

*Cardiac decompensation.....	5.00
Acute nephritis.....	5.00
Sepsis.....	5.25
Pleurisy with effusion.....	5.35
Secondary anaemia.....	5.37
Arteriosclerosis.....	5.37
Chronic glomerulo-nephritis with cardiac decompensation.....	5.38
Pernicious anaemia.....	5.45
Appendicitis.....	5.62
[Acute endocarditis.....	5.63]
Chronic glomerulo-nephritis without cardiac decompensation.....	5.70
Typhoid fever.....	5.85
Carcinoma.....	6.00
Normal cases.....	6.03
[Peptic ulcer.....	6.04]
[Lobar pneumonia.....	6.17]
[Aplastic anaemia.....	6.52]
[Syphilis.....	6.56]
[Diabetes mellitus.....	6.65]

Only a few cases were observed in the groups with brackets. The average of 57 cases in which there was either nephritis or cardiac disease or both is 5.33. This amounts to a five-fold increase in the acidity of the urine.

* Including a number of observations not reported in this paper.

These measurements appear to establish certain facts:

1. The variations in hydrogen ion concentrations of urines are on the whole such as have been previously reported. Normal urine seems to range from a concentration of ionized hydrogen of $abc \cdot 4.82$ to one of about 7.45. The mean value is almost exactly 6.00. The earlier investigation of one of us is thereby confirmed, but a wider variety of "normal" cases and of conditions discloses a greater variation and a slightly higher mean.

2. Pathological conditions cause occasionally an even greater acidity than we have ever observed normally, but in our experience never unusual alkalinity. Commonly however the range is within normal limits.

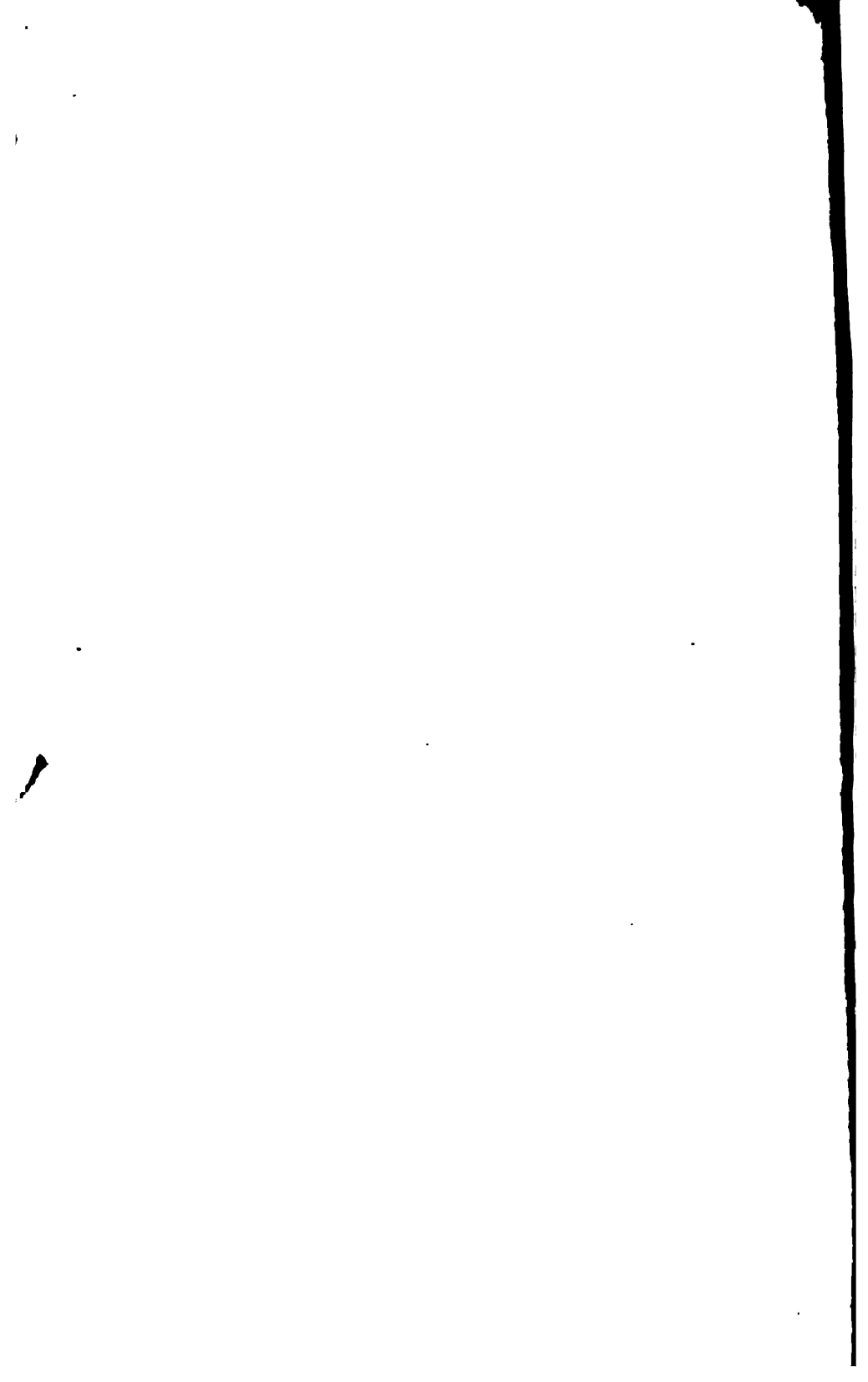
3. The mean acidity in cardio-renal cases is undoubtedly high—5.33—corresponding to an actual concentration of hydrogen ions 50×10^{-7} , instead of 10×10^{-7} , the normal mean. Such disparity is almost certainly significant and suggests the common

occurrence of a condition which other observations lead us to consider a form of acidosis.

4. Various other pathological conditions appear to be commonly accompanied by unusual acidity of the urine but, in view of the normal variations and the consequent need of large numbers of observations, we do not at present feel justified in attempting to classify them.

5. In those pathological conditions of which we have thus far statistics in numbers sufficient to justify a conclusion, the mean value of the acidity of the urine appears never to fall below that of normal urine.

We wish to thank Dr. Frederick C. Shattuck for making this work possible, and Dr. W. F. Boos for his kindness in permitting the use of the Chemical Laboratory. Thanks are also due the members of the visiting staff and others of the Massachusetts General Hospital for their kindness in supplying clinical material.



THE PROBLEM OF ENZYME SYNTHESIS. I.

LIPASE AND FAT OF ANIMAL TISSUES.

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Since the demonstration of the reversibility of enzyme action by Croft Hill,¹ Kastle and Loevenhart,² Pottevin,³ Hamsick,⁴ Taylor⁵ and others, the general correspondence between enzyme reactions and those catalyzed by inorganic substances has been conceded. The true catalytic nature of the enzymes seems beyond question. Where this correspondence appears to be lacking in certain details, as in the hydrolysis of esters by lipase, we are justified in conceiving other factors operative in checking or destroying the enzyme before the reaction has a chance to attain equilibrium. Under proper conditions therefore it seems probable that all hydrolytic enzymes should be capable of synthesizing the substances which they hydrolyze under other circumstances. The broad biological significance of this fact was pointed out by Loevenhart in 1902 in his paper dealing with lipase and lipogenesis.⁶ The theory there advanced offered biologists a most attractive explanation of the chemical mechanism involved in the digestion, absorption, transportation, deposition and utilization of fats. The data presented in support of the theory seemed adequate to convince one of its tenability, and the fact that it has been so widely accepted throughout the scientific world indicates the general applicability of the conception. Indeed it may be said that the theory of enzyme

¹ Croft Hill: *Journ. Chem. Soc.*, lxxiii, p. 634, 1898.

² Kastle and Loevenhart: *Amer. Chem. Journ.*, xxiv, p. 491, 1900.

³ Pottevin: *Compt. rend. Acad. des Sci.*, cxxvii, p. 1152, cxxviii, p. 378, 1903.

⁴ Hamsick: *Zeitschr. f. physiol. Chem.*, lix, p. 1, lxx, p. 232, 1909.

⁵ Taylor: *this Journal*, iii, p. 87, 1907.

⁶ Loevenhart: *Amer. Journ. of Physiol.*, vi, p. 331, 1902.

synthesis of tissue compounds is the only explanation generally held and taught today to account for the building up of protein, fats and carbohydrates.

The work upon the synthesis of proteins *in vitro* through the agency of proteolytic enzymes, has not proven adequate to the task as yet, but the complexity of the reactions involved and the lack of those modifying influences which undoubtedly exist in the living cell are sufficient to account for the failures up to the present time. But while no one has succeeded in building up a typical native protein, great success has attended the efforts toward the synthesizing of carbohydrates and fats. The work of Hamsick⁷ in particular may be cited, for he has succeeded in demonstrating a 30 per cent synthesis of triolein from its components. He has further shown that the presence of bile salts is important in facilitating the synthetic reaction just as the hydrolytic. It is not necessary to multiply examples of enzyme syntheses *in vitro*, since the fact is too well established to need further proof. Attention should be called in passing, however, to the conditions under which successful reversions take place. The absence of water from the reacting mixture is a prime necessity. Croft Hill⁸ used a 40 per cent dextrose solution in his synthesis of isomaltose—a concentration far above anything found in vegetable sap, so far as we know. Hamsick used pure glycerine, oleic acid and dry pancreas powder in accomplishing his notable syntheses. The reason for the small yield of ethyl butyrate obtained by Kastle and Loevenhart was undoubtedly the dilution of the reacting mixture. A small active mass of water is as important in syntheses catalyzed by enzymes as in those catalyzed by mineral acids for example. Concentrated sulphuric acid, pure fatty acids and alcohols are commonly used in laboratory ester syntheses, because under these conditions the mass of water is kept at a minimum and the hydrolytic reaction is correspondingly small. It is well known that a mixture of ester, water and mineral acid tends toward hydrolysis rather than toward the reverse reaction so long as the water is above a certain proportion. It has been shown⁹ similarly that pancreatic lipase tends toward the complete hydrolysis of

⁷ Hamsick: *loc. cit.*

⁸ Croft Hill: *loc. cit.*

⁹ Bradley: this *Journal*, viii, p. 251, 1910.

triolein if water is present to the extent of 50 per cent of the reacting mixture, and the more water present the more rapid is the hydrolysis. If less than 50 per cent of water is present equilibrium is reached before hydrolysis is complete. It requires, however, a relatively small amount of water to secure a relatively large degree of hydrolysis and conversely a high degree of desiccation is necessary to produce noteworthy syntheses. This fact, however, does not exclude the possibility of enzyme syntheses in cells where the total water content approximates 80 per cent, since there is abundant evidence of the fact that concentrations in localized portions of the cell may be far greater than in other adjacent portions. The probable foam-structure of cytoplasm, the adsorption phenomena at the boundaries of different phases, gives us a mechanism which appears adequate to explain why at one point in a cell fat may be synthesized from its components, while at another point it may be undergoing hydrolysis at the same time. Such data as have been presented by Macallum on the localization of potassium and other salts in growing structures, may be cited as proof of the possibility of attaining high concentrations of soluble and diffusible substances in sharply localized areas of the cell. The familiar formation of excretory vacuoles in the unicellular organisms, where water charged with products of metabolism collects and grows, moves to the periphery of the cell and discharges into the surrounding medium, is a striking example of this phenomenon.

So far therefore it seems quite possible to visualize in a crude way a mechanism by which syntheses by means of enzymes can take place in cells whose water content as a whole is too high to admit of this result once the cell structure is lost or disturbed. The theory requires a complexity of structure and reaction which is abundantly borne out by other facts. The theory has, however, never been given that close scrutiny which so broad a working hypothesis deserves. Very little has been done to establish it beyond proving the true catalytic nature of enzyme reactions. It has therefore seemed advisable to secure data upon this important phase of metabolism, confirming or disproving the theory as the case may be. At the outset it has been evident that the methods of actually scrutinizing tissue syntheses are difficult and uncertain of interpretation, and that no single reaction or series

of reactions from a single point of view can be found sufficient to the task unless the results are clearly positive. Where results are equivocal or negative the question must still remain open, because of the complexity of the reacting media and the sensitiveness of enzyme reactions in general. It was hoped, however, that by attacking the problem from a variety of angles enough data might be secured to give basis for the ultimate acceptance of the hypothesis upon more than a hypothetical basis or for its rejection as inadequate to explain the facts.

Before attempting a critique of the theory it might be well to state it in the terms generally accepted by biologists. For purposes of defining the problem I have taken the presentation of the theory given by Wells in his *Chemical Pathology*,¹⁰ "All metabolism, then, may be considered as a continuous attempt at establishment of equilibrium by enzymes, perpetuated by prevention of attainment of actual equilibrium through destruction of some of the participating substances by oxidation or through other chemical processes, or by removal from the cell or entrance into it of materials which overbalance one side of the equation." In other words the intracellular enzymes which are present in every living cell, lipase, diastase, proteases, etc., function as hydrolytic and as synthetic agents. Under certain conditions, dependent upon the active masses of the compounds involved, fats, carbohydrates and tissue proteins may be built up and stored in the cell. Under other conditions, these same substances will be hydrolyzed to simpler compounds as an initial step in utilization and catabolism. All of the strictly reserve deposits of fat, carbohydrate and protein are assumed to be built up in this way in times of nutritional abundance. The store of glycogen in the liver following a carbohydrate-rich meal, the deposits of starch and oil in seeds, the laying on of adipose tissue in animals, may be instanced as cases in point. All such deposits are subsequently hydrolyzed and removed as conditions of nutritional want are experienced. The glycogen quickly diminishes in the liver of the starving mammal, the oil and starch undergo hydrolysis as the seed germinates and the fatty tissues diminish in the mammal to maintain bodily heat and energy. The diastase of the liver, lipase and diastase of the seed, the lipase of active adipose tissue,

¹⁰ H. G. Wells: *Chemical Pathology*, p. 68.

are already well known. The proteases of cells, which after death accomplish the digestion of the cell proteins, during life may function in both hydrolysis and synthesis, and thus be responsible for the building up of the characteristic tissue proteins. Why the proteases of one cell build up muscle proteins and those of another connective tissue we cannot explain except upon the ground of specificity of enzymes and the complex of conditions existing in different cells exerting a directive action upon the steps involved in synthesis.

In a general way, then, we find cells equipped with enzymes capable of performing their synthetic reactions. Also, but to a less striking degree, those cells which are most actively engaged in the synthesis of a given compound seem to be equipped with more of the necessary enzyme than are other cells less actively engaged in this particular type of metabolism.

There are many exceptions already known to this statement, but they are generally assumed to be simply exceptions in no way invalidating the general hypothesis. It is certain that a general correlation between the lipase of tissues and their activity in fat metabolism, as expressed by their fat content in periods of plenty, would do much to strengthen our belief in the theory. At the same time fat content alone is not an indication of the fat metabolism of the organ; vascularity, lymph supply and other indications of the activity of an organ must be taken into consideration. In homologous organs, such parallelism however would be in the nature of positive evidence in favor of the hypothesis. On the other hand, lack of such parallelism would not necessarily weaken the theory, because of the presence of so many unknown factors in the cell itself. Lack of such parallelism would however weaken the force of any argument based upon its previous assumption. We have chosen the distribution of fat and lipase as our first point of inquiry chiefly because in Loevenhart's original work on the subject a certain degree of parallelism was found. Thus, active mammary gland was more active lipolytically than resting and subcutaneous adipose tissue was more active than muscle or kidney fat. It seemed desirable to extend these observations over a much wider field and to determine whether in a broad sense there is any relation between fat content and lipase activity.

A preliminary examination of a considerable number of tissues secured from a wide variety of species of marine animals was made

at Woods Hole in 1911. Lipase was estimated in the usual way by making up a definite weight of tissue "Brei" to a standard volume, adding a soluble ester, ethyl butyrate, and titrating the acidity developed. After a period of three days the lipase reaction has usually reached equilibrium, so that the acidity at the end of that time represents roughly the amount of lipase present. Fat was extracted from another weighed portion of the fresh tissue by boiling alcohol and ether. The extracts dried at 105° were reextracted with ether, evaporated in a weighed capsule and dried to constant weight. The results from this preliminary survey are not considered accurate, but they are sufficiently so to indicate that a correlation between lipase and fat content in the tissues examined does not exist. Some of the tissues richest in fat are poorest in lipase, and *vice versa*. The following table contains a few of these results.

TABLE I.

NO.	TISSUE	FAT FROM 5 GRAMS "BREI"	ACIDITY DEVELOPED BY 5 GRAMS "BREI" IN 100 CC. WATER, 2 CC. ETHYL BUTYRATE
		grams	cc.
1	Shark liver.....	2.34	0.30
2	Dog fish liver.....	2.28	0.50
3	Dog fish liver.....	2.19	1.40
4	Shark liver.....	2.16	0.20
5	Shark liver.....	1.39	0.40
6	Dog fish liver.....	1.15	0.90
7	Flounder liver.....	1.09	2.00
8	Squeteague liver.....	0.84	0.85
9	Limulus liver.....	0.16	0.70
10	Shark sperm.....	0.10	0.40
11	Shark kidney.....	0.06	0.30
12	Shark pancreas.....	0.03	0.60
13	Limulus blood.....	0.02	0.30

It will be seen that while the lipase content of shark liver and Limulus blood is exactly the same, the fat of the former is one hundred times greater than that of the latter. Again, No. 6, dog fish liver, contains one-half the fat that shark liver, No. 1, contains but its lipase number is three times as great. Flounder liver contains less than one-half the fat of shark liver but seven times the amount of lipase.

A comparison of such figures as those in Table I is not however satisfactory. The lipase is presumably associated with the protoplasmic portion of the cell contents—the dry, fat-free proteins. A comparison of shark liver, which is about 50 per cent fat, with other tissues, which contain little or no fat, on a basis of fresh tissue weight is obviously incorrect. The real protoplasmic basis of fresh shark liver must be much less than that of mammalian liver, for example, and a comparison of the lipolytic activity of the two would appear to make the mammalian liver tissue more active than it actually is. Furthermore the introduction of the insoluble ester triolein along with the shark liver “Brei” must introduce a further error of comparison the extent of which we do not know. In comparing aliquots titrated with $\frac{N}{10}$ NaOH and $\frac{N}{10}$ KOH (alcoholic), it was found that the acidity in the alcoholic titration was frequently nearly double that of the water solution. Evidently the lipase acts on both the soluble and the insoluble ester at the same time and at approximately the same rate. If there were no fat introduced along with the “Brei” it would seem reasonable to assume that the ethyl butyrate would be hydrolyzed more rapidly. It is fair to state however that, after making all possible allowance for this latter factor, the general results would remain quite unmodified.

A second, more accurate investigation was made, with a view to determining what corrections should be made on the basis of protein or protoplasmic contents of the tissues examined.¹¹ Fat was estimated, the dry weight of the tissues, and the lipase activity. Calculating the dry, fat-free weight of the tissues it is seen that a wide range exists—from 4 to 40 per cent. We may assume that 20–25 per cent represents about the average. Then a tissue like shark liver which may contain as little as 4 per cent fat-free substance, represents only about one-fifth or one-sixth of the amount of active tissue found, for example, in porpoise liver. As has been shown, the law of Schütz holds approximately through a considerable range of enzyme concentration: the height of digestion varies with the square root of the enzyme, when equilibrium is allowed to be attained. Accordingly four times the enzyme present should double the acidity developed at equilibrium.

¹¹ I wish to acknowledge the valuable assistance of Mr. Eugene Kellersberger in securing these data.

TABLE II.

NO.	SPECIES	TISSUE	FAT	RESIDUE	PROTEIN	N % ACID PER 5 CC. TO ALIQUOT	CORRECTION FACTOR	CORRECTED ACIDITY NUM- BER
			per cent	per cent	per cent	cc.		
1	Porpoise.....	liver	5.06	26.44	21.38	9.20		9.20
2	Tautaug.....	liver	49.20			4.60		4.60
3	Pogy.....	liver	13.37			4.00		4.00
4	Dog fish.....	liver	39.35	52.58	13.23	1.80	2	3.60
5	Dog fish.....	liver	34.69			3.15		3.15
6	Shark.....	liver	63.14	66.52	3.38	0.50	7	3.50
7	Shark.....	liver	62.58	66.52	3.94	0.40	6	2.40
8	Skate.....	liver	12.50			2.30		2.30
9	Skate.....	liver	16.45			2.10		2.10
10	Shark.....	liver	45.49	56.45	10.96	0.65	2	1.30
11	Shark.....	liver	45.59	56.78	11.19	0.65	2	1.30
12	Dog fish.....	liver	29.72	50.56	20.84	1.25		1.25
13	Shark.....	liver	58.12	65.86	7.74	0.40	3	1.20
14	Shark.....	liver	37.70	49.88	12.18	0.60	2	1.20
15	Dog fish.....	liver	17.24			1.15		1.15
16	Shark.....	liver	51.58	60.70	9.12	0.50	2	1.00
17	Sycotypus....	liver	7.91	32.97	25.06	1.70		1.70
18	Sycotypus....	liver	5.23	29.97	24.74	1.00		1.00
19	Star fish.....	dig. gland	15.78	36.03	20.25	1.00		1.00
20	Limulus.....	dig. gland	2.83	13.48	10.65	0.30	2	0.60
21	Limulus.....	muscle	0.79	21.09	20.30	1.40		1.40
22	Tautaug.....	muscle	0.90	20.42	19.52	1.20		1.20
23	Tautaug.....	muscle	0.90			0.90		0.90
24	Sycotypus....	radula mus.	1.62			0.65		0.65
25	Sycotypus....	pedal mus.	0.95	26.89	26.00	0.50		0.50
26	Skate.....	muscle	0.60			0.50		0.50
27	Limulus.....	muscle	0.76	13.85	13.09	0.50	2	1.00
28	Skate.....	muscle	0.78	31.71	20.29	0.40		0.40
29	Sycotypus....	pedal mus.	0.78	24.67	23.89	0.30		0.30
30	Pogy.....	red mus.	8.80	28.13	19.33	0.30		0.30
31	Dog fish.....	muscle	1.25			0.25		0.25
32	Pogy.....	white mus.	1.33	22.00	20.67	0.20		0.20
33	Dog fish.....	muscle	0.70	21.38	20.68	0.15		0.15
34	Dog fish.....	white mus.	1.01	23.80	22.79	0.00		0.00
35	Dog fish.....	red mus.	2.65			0.20		0.20
36	Shark.....	muscle	0.74			0.00		0.00
37	Shark.....	heart mus.	2.93			0.25		0.25

TABLE II—Continued.

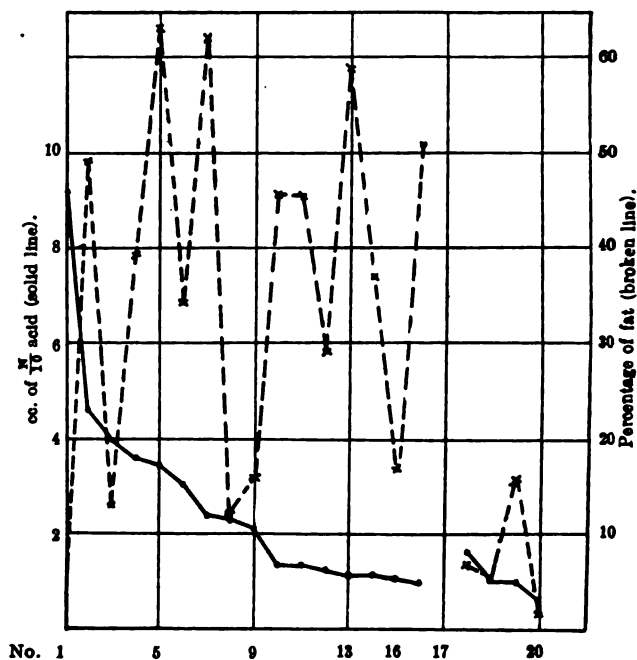
NO.	SPECIES	TISSUE	FAT	RESIDUE	PROTEIN	N ACID PER 5 CC. TO ALIQUOT	CORRECTION FACTOR	CORRECTED ACIDITY NUM- BER
			per cent	per cent	per cent	cc.		
38	Limulus	eggs	8.29	49.00	40.71	1.50	$\frac{1}{4}$	0.75
39	Limulus	eggs	8.90	48.95	40.05	1.30	$\frac{1}{4}$	0.65
40	Skate	eggs	6.17	33.15	26.92	0.70		0.70
41	Star fish	eggs	3.38	11.49	8.11	0.30	3	0.90
42	Skate	ovaries	3.92			1.60		1.60
43	Skate	album. gland	2.59	34.58	31.99	1.00		1.00
44	Shark	testis	3.42			4.10		4.10
45	Tautaug	testis	2.92			0.90		0.90
46	Shark	spleen	2.31			2.30		2.30
47	Shark	spiral valve	2.40			0.90		0.90
48	Limulus	blood	0.00			0.90		0.90
49	Limulus	blood	0.01			0.45		0.45
50	Shark	blood	0.24			0.15		0.15
51	Sycotypus	blood	0.31			0.00		0.00
52	Sycotypus	blood	0.02			0.00		0.00

Since this proportionality is not a strict one and only holds through a limited range of variation of the enzyme, we have decided to give every possible advantage to the tissues, poor in protoplasmic residue, in the tabulated comparisons. We have therefore assumed a direct or linear relation between enzyme and acid developed. As will be seen in the preceding table, even this excessive correction allowance fails to account for the differences which we find between homologous organs of different species, nor does it greatly lessen the striking lack of correlation between fat and lipase.

Graphs have been prepared to show the relationship between lipase and fat, segregating the figures for muscle, liver, digestive glands, eggs, etc. The lack of parallelism is quite evident.

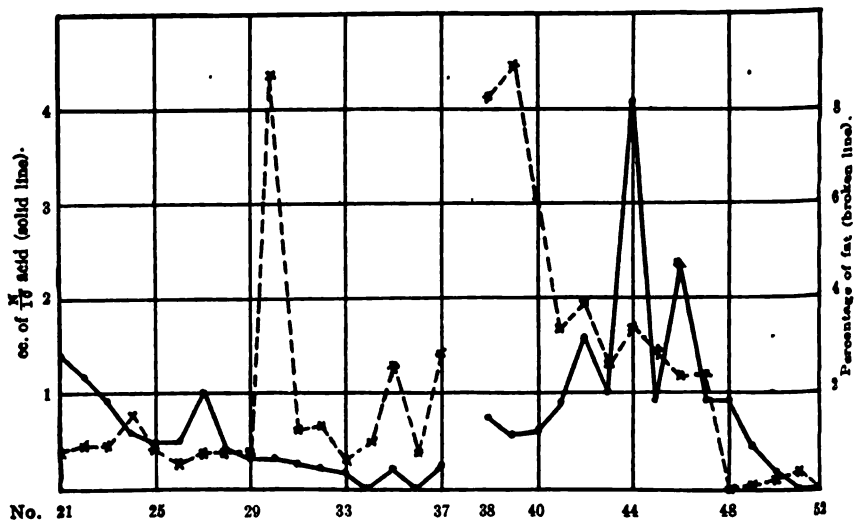
It may be suggested that the comparison of tissues from widely different species of animals is hardly a fair one. The function of the liver of the teleost must be very different quantitatively at least from that of the mammal. Shark and dog fish livers are always found rich in fat, while mammalian liver is uniformly poor. But a series of figures limited to sharks and dog fishes still shows no parallelism between the fat and lipase.

LIVER TISSUE.



MUSCLE.

MISCELLANEOUS TISSUE.



In the following table are presented some miscellaneous figures,¹² which cannot be strictly compared with those of preceding tables since the digestions were carried on at 37° instead of 25°.

TABLE III.

SPECIES	TISSUE	N 10 ACIDITY PER 5 CC. ALIQUOT	FAT
		cc.	per cent
Cat.....	liver	7.80	7.01
Rabbit.....	liver	6.40	4.00
Dog.....	liver	6.20	3.23
Perch.....	liver	5.00	4.52
Shad.....	liver	3.40	13.00
Calf.....	lung	4.80	2.44
Beef.....	kidney	4.00	2.17
Calf.....	heart muscle	2.90	2.27
Shad.....	spermatoid	2.70	2.40
Cow.....	mammary (active)	1.90	11.00
Goat.....	mammary (active)	1.50	4.30
Calf.....	brain	1.10	4.72
Goat.....	blood	0.80	0.12
Dog.....	thoracic lymph	0.07	0.78

Attention should be called to the interesting observation, which I have frequently confirmed, that active mammary tissue is less rich in lipase than mammalian liver, and even than heart muscle, beef kidney and calf lung, although it is richer in fat than these tissues and is daily secreting very large amounts of fat in the milk, in addition. Instead of being unusually rich in lipase on account of its active secretion of fat, it is found to be about on a par with such other tissues as fish ovaries and testes, spleen, brain and other gland structures of mammals and invertebrates. Compared with a large number of tissues active mammary gland is not a tissue rich in lipase; it is only twice as active as blood itself. The fact that active mammary tissue is richer in lipase than inactive, as was pointed out by Loevenhart, is probably due in large measure to its hyperplastic condition during lactation. It is certain that the secreting epithelial cells are far more abundant in the active gland than in the resting one, so that a given weight of active gland tissue must contain a far greater proportion of gland cells and

¹² Data collected by Clarence Schuldt for graduation thesis. 1912.

correspondingly less connective tissue than the same weight of resting mammary. Not knowing the relative proportion of epithelial cells in the two types of tissues it is impossible to estimate how much of the increased lipolytic activity of the secreting gland is due to increased metabolic activity of the cells themselves, and how much to their numerical increase.

CONCLUSIONS.

1. No broad correlation exists between the fat and lipase content of tissues.
2. Homologous organs in allied species, such as teleost livers, fish muscles, etc., show no parallelism between fat and enzyme.
3. Some of the most active fat-producing tissues are relatively poorer in lipase than many other tissues which never normally contain or produce more than a small percentage of fat. Active mammary tissue affords the most striking example of this when compared with lung, kidney and muscle tissues.
4. Quantitative comparison of fat and lipase in animal tissues gives no positive evidence in support of the theory of enzyme synthesis.

THE PROBLEM OF ENZYME SYNTHESIS. II.

DIASTASE AND GLYCOGEN OF ANIMAL TISSUES.

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(Received for publication, November 2, 1912.)

In a recent paper by Hugh MacLean,¹ diastatic activity of mammalian tissues was reported to have the following order: kidney, lungs, liver, heart, stomach and bladder and skeletal muscles. There were individual and species variations found, but the average diastatic activity was in the order named. It is interesting to note that kidney and lung tissues which are most active, are seldom found to contain more than traces of glycogen, if any. Muscles, which are least active diastatically, frequently are quite rich in glycogen and normally contain some of that carbohydrate. MacLean pointed out the lack of correlation between glycogen storage and diastatic activity in the tissues examined.

We have extended this field of inquiry to include a number of lower forms known to be rich in glycogen. For example the muscle tissues of molluscs are usually very rich in glycogen, so much so indeed that it makes one of the best sources for the preparation of large amounts of this substance. Are such muscles proportionately rich in diastase?

MacLean's method of preparing the tissues for examination was followed. The living tissue was ground fine into a large excess of alcohol and kept thoroughly mixed. The alcohol was changed several times in twenty-four hours, pressing the pulp dry each time in a meat press. At the end of this period the tissue was washed quickly with ether and gasoline to remove alcohol and fats, pressed dry and spread out in thin layers on filter paper in a current of air. The dry pulp was re-ground and sieved. Two grams of this powder, 25 cc. of a stock starch solution used throughout the experiments and a little toluene were made up to 100 cc. The time

¹ MacLean: *Biochem. Journ.*, iv, p. 467. 1909.

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required for the disappearance of the starch and the erythro-dextrin reactions was used as the indication of the activity of the diastase present. We have also used glycogen solutions for digestion but have found such complete parallelism between the starch and glycogen digestions that the former has been adopted in our routine examinations. It is believed that for comparative purposes, such as these experiments require, the change to achroë-dextrin, as indicated by the iodine reaction, is as valuable as a quantitative determination of sugar at the end of a fixed time.

Glycogen was estimated in a weighed sample of the tissue powder by the Pflüger method, boiling with strong alkali for two hours, precipitating with alcohol, redissolving and reprecipitating several times, and finally weighing the purified substance dried to constant weight. Where traces only could be found they were used for qualitative reactions for the identification of the substance as glycogen. In a few cases where only a limited amount of the tissue could be obtained the fresh "Brei" was used for the detection of diastase.

NO.	SPECIES	TISSUE	DRY WEIGHT	TIME	GLYCOGEN	REMARKS
			grams		per cent	
1	Pecten.....	liver	2	37 min.	trace?	
2	Sycotypus....	liver	2	47 min.	1.15	
3	Rat.....	liver	2	60 min.	trace	
4	Limulus.....	liver	2	90 min.	none	
5	Sand shark....	liver	2	240 min.	none	
6	Sand shark....	liver	2	480 min.	none	
7	Limulus.....	eggs	2	570 min.	2.57	
8	Limulus.....	muscle	2	2½ day	trace	
9	Dog fish.....	liver	2	5 days	1.35	
10	Pecten.....	muscle	2	5 days?	4.62	erythro-dex.*
11	Squid.....	muscle	2	5 days?	none	
12	Dog fish.....	muscle	2	6 days?	0.20	
13	Sand shark....	muscle	2	7 days?	none	
14	Squeteague....	muscle	2	7 days?	trace	
15	Lobster.....	muscle	2	7 days?	none	
16	Sand shark....	muscle	2	8 days?	none	
17	Sycotypus....	muscle	2	8 days?	3.78	blue-purple.
18	Swordfish.....	muscle	2	?	none	

* Where a question mark appears it indicates that no sure indication of digestion could be detected at the end of the time given to that experiment. In the two cases noted, Nos. 10 and 17, there was some slight indication of change in the starch solution, though in 17 it was not clear whether it was due to digestion of starch or not.

NO.	SPECIES	TISSUE	FRESH WEIGHT	TIME	GLYCOGEN	REMARKS
			<i>grams</i>			
19	Sycotypus....	muscle	10	8 days ?	0.68	
20	Shark.....	muscle	10	3 days	trace	
21	Sycotypus....	blood	75	3 days	none	
22	Squid.....	liver	3	5 days	none	
23	Squid.....	blood	25	3 days	none	
24	Squeteague...	liver	3	3 hours	trace	
25	Lobster.....	liver	25	3 hours	trace	
26	Lobster.....	blood	25	?	none	
27	Sycotypus....	liver	10	3 hours	none	
28	Shark.....	liver	10	5 hours	none	

An analysis of the results shows in the first place that the treatment to which the tissues are subjected in drying and rendering them fat-free, does not greatly alter the diastatic activity; compare 2 and 27; 4, 6 and 28. It is improbable therefore that, by this treatment, diastase has been destroyed in any of the tissues which fail to digest starch.

In the second place, the most active tissues diastatically, *Pecten*, *Sycotypus*, rat, *Limulus*, and shark livers and *Limulus* eggs, do not all contain glycogen. It is quite probable however that in periods of nutritional plenty these tissues might all contain glycogen, since with one exception they are livers or digestive glands which are believed to function like the liver in storing glycogen. On the other hand, the least active tissues are the muscles. With the exception of *Limulus* muscle (which contains no glycogen) these tissues are practically devoid of diastatic enzymes. Yet *Pecten* and *Sycotypus* muscles are the richest tissues which we have analyzed for glycogen. In the case of *Pecten* muscle there was an unmistakable change to erythrodextrin, but during the five-day digestion period there seemed no further change. At the end of an eight-day period *Sycotypus* muscle showed no more change than expressed by a slight purple tint in the digestion mixture when treated with iodine, instead of the original clear blue. A control of 10 grams of the perfectly fresh muscle "Brei" showed no digestion at the end of eight days. Inasmuch as *Sycotypus* blood showed diastase, it is not surprising that the muscle sample 17 appeared to have a trace of activity. Indeed it might be considered more surprising that the tissue showed so

little. When the gasteropods were killed, however, the large blood spaces of the pedal muscle were cut in several directions to allow free bleeding, and the muscles themselves under this stimulus contract to the utmost, thus squeezing out practically all of the blood present. What extremely slight digestion appeared to go on in 17 we may attribute to a trace of blood rather than to the muscle fibres themselves.

It should be noted that our figures disprove the statement of Abderhalden,² that: "With the gasteropods the liver is the only place in which glycogen is deposited to any extent; in the other organs the amount is hardly worthy of consideration." The gasteropod, *Sycotypus canaliculatus*, regularly contains large amounts of glycogen in the pedal muscle. The specimens here examined had been kept in captivity for many weeks, long enough to remove most of the glycogen from the liver in several individuals examined and to have reduced the percentage present in the muscles. Freshly dredged specimens in good condition will yield much more glycogen than the figures above would indicate. The same is true of the muscles of many lamellibranchs, of which Pecten is an example.

In confirmation of MacLean's work, we find that tissues rich in diastase may or may not contain glycogen; and, what is far more significant from the point of view of the enzyme-synthesis theory, tissues rich in glycogen may or may not contain diastase. While it is easy to conceive of a tissue rich in an enzyme failing to build up the synthetic product of that enzyme's activity, it is not so easy to explain the absence of an enzyme from a tissue regularly building up a certain compound, if we attribute that synthesis to the enzyme. At the same time the absence of the enzyme does not invalidate the general theory. It is possible that the enzyme has disappeared after the synthesis; or that the diastase of the blood is capable of diffusing into the cells at certain times; or that in an animal whose whole metabolism is on so low a level, an amount of diastase too small to detect is still sufficient to synthesize and hydrolyze the glycogen stored in the pedal muscle. It is not impossible of course that glycogen is synthesized in the active liver or "hepato-pancreas" and is transported thence to the muscle tissue in the blood. The presence of glycogen in the latter might

² Abderhalden: *Text-book of Physiological Chemistry*, p. 46.

be too slight also for detection at any given time. There are many considerations which render the negative results, recorded above, equivocal rather than negative of interpretation. It is clear however that the experiments do not add anything to substantiate the theory.



THE PROBLEM OF ENZYME SYNTHESIS. III.

DIASTASE AND STARCH OF PLANT TISSUES.

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(Received for publication, November 2, 1912.)

It is well known that diastase is widely distributed in the active tissues of plants. Just what its relative strength is in different tissues has been less carefully determined. Furthermore the quantity of the enzyme is variable so that only very general statements can be made concerning its presence. The season of the year, the condition of the plant and its stage of development, etc., all exercise an influence in determining the activity of the enzyme. For this reason much of the data collected upon this question was unavailable for the purposes of this investigation, and a series of determinations was therefore made to determine whether a relationship exists in the plant tissues between starch and the enzyme which is believed to synthesize it from the more soluble carbohydrates. A definite correlation between the enzyme diastase and the starch content would throw much light on the general question of enzyme synthesis. The plant should be especially favorable material for this problem because of the relatively simple metabolism there found.

The method used was that described in a previous paper for the detection and estimation of diastase in animal tissues. The plant organs were not treated with alcohol and ether since few plant tissues contain appreciable amounts of fat. Five grams of the finely pulped material were weighed out, 25 cc. of the same starch solution added in all cases, toluene and water to make the volume 100 cc. The iodine reaction was used as the test of digestion, and the time required to convert the starch solution to the achroödextrin stage indicated in a rough way the activity of the diastase present. A number of tissues were assayed for their dry weight as a further check on the interpretation of the figures of digestion.

A number of objections may be urged at the outset. First: The method is not a quantitative one; the end point with iodine is not sharp and a considerable error is thereby introduced. Making all possible allowances for error in this direction does not however invalidate the general results—a given tissue is ten or a hundred times more active than another.

Second: Many tissues already contain starch in larger or smaller amounts, and the presence of this starch must inevitably alter the time required for digestion to the achromic point. This error is in many cases a serious one. In many others however the starch grains are so resistant to the diastase that filtering the samples taken for tests removes them, and the solution then represents the original starch solution added. We have found a number of tissues where the reaction of the starch grains in suspension persisted hours or even days after the soluble starch solution had all been converted into dextrins which gave no iodine color reaction.

Third: Very noticeable differences exist between the action of diastases of different origins. In some cases erythrodestrin is rapidly formed from the starch, and then persists for long periods of time. In others no erythrodestrin reaction develops; the solution remains blue-reacting to the end, and merely decreases in viscosity to the vanishing point. In others the steps from starch to erythrodestrin to achrodestrin are well defined and follow each other in approximately the same time. This difference is possibly an indication of a complex of enzymes in what is called "diastase," the factor of which hydrolyzes starch, another erythrodestrin, and in a given tissue "Brei" the erythrodestrin-digesting enzyme is much more abundant than the starch-digesting, then the speed of the entire reaction will be expressive of the speed of the initial step of hydrolysis. The reddish color at the same time never be appreciable, since the erythrodestrin will be hydrolyzed as rapidly as formed. In other cases, where the initial step is rapidly taken but the dextrinase is wanting, the mixture rapidly assumes the red-staining stage and then very slowly or never passes to the achromic point.

Sections of the organs were stained with iodine, and where the reaction was negative or equivocal, samples of the "Brei" were taken with boiling water and the extract tested for starch.

TABLE I.

SPECIES	TISSUE	DRY WEIGHT	DIGESTION TIME	STARCH CONTENT
		<i>per cent</i>	<i>hours</i>	
Green pea.....	leaf		0.70	None.
	pod and seeds	12.4	0.50	Small amount.
	seeds, medium	24.6	0.50	Medium.
	pod, medium	13.2	0.80	Small amt. dextrin.
	seeds, mature	80.0	1.00	Abundant.
Lima bean.....	seed, medium	23.2	6.00	Abundant.
	pod, medium		5.00	Small amt. dextrin.
	leaf	15.4	0.50	Small amt. dextrin.
Kidney.....	seed, medium	27.6	5.50	Medium.
	pod, medium	14.8	5.50	Abundant dextrin.
	leaf	18.0	0.25	Small amt. dextrin.
Kohlrabi.....	root		1.50	None.
	leaf		1.50	None.
Onion.....	root, young	13.4	?	None.
	leaf, young	10.6	48.00	None.
Bayberry.....	leaf		72.00	None.
	berry, mature		72.00	None.
Radish.....	root, mature	5.6	0.70	None.
	leaf, mature	8.6	0.70	None.
Carrot.....	root, medium	10.8	2.50	Abundant.
	leaf, medium		22.50	None.
	root, young		?	None.
Red beet.....	leaf, young		1.25	None.
	root, mature		14.00	None.
	leaf, mature		6.00	None.
Mangel.....	root, mature		?	None.
	leaf, mature		7.00	None.
	root, medium		18.00	Some dextrin.
Potato.....	tuber, medium		17.00	Very abundant.
	leaf, medium.		0.75	None.
	seed, medium		7.50	Abundant.
	husk, medium		6.00	None.
Corn.....	seed, mature		6.50	Abundant.
	cob, mature		6.50	Small amount.
	seed, young		5.00	Medium amount.
	cob, young		6.50	Small amount.
	husk, young		2.00	None.
Marrow squash	seed, mature	25.2	1.25	Medium.
	pulp, mature		?	Abundant.
	pulp, young		48.00	Abundant.
Cantaloupe ...	leaf, mature		0.50	None.
	pulp, mature		72.00	None.
	seed, mature		?	Small amount.

In the table the relative amounts of starch found are indicated. In general it is a fact that leaves contain starch. In some it is found always, in others only while photosynthesis is in rapid progress. The fact that our plant tissues were usually secured early in the morning, before photosynthesis had gone on to any large extent, is probably the explanation of our failure to find starch in many of the green parts of plants. During the night, the starch, in such parts as the leaves, usually disappears. It is believed to be converted into sugar and removed by the sap to the organs of storage.

About one hundred tissues were examined in this way. The results are very divergent. A few of the more striking figures are given in the table below. The publication of the rest would not add materially to the solution of the present problem.

The above typical results, together with the mass of data unpublished, shows the following conclusions are warranted:

First: Diastase is as a rule most abundant in the leaves, where starch is never stored permanently, but where it may be found in small amounts during photosynthesis. Leaves of different species exhibit very marked differences in their diastase content. The leaves of the bayberry, onion and leek contain so little of the enzyme as to be doubtful. On the other hand the leaves of the legumes were among the most active tissues examined. It is of interest to note that the leaves of the legumes were usually found to contain considerable starch or a dextrin-like compound, though there were several exceptions noted.

Second: No general deductions can be drawn relating the diastatic activity of a tissue with its starch-storing function. We find plants like the beets and mangels, which store no starch in the root but instead store sugar, having a highly active leaf and no diastase in the root. Other plants like the radish and rutabaga and kohlrabi, which also store sugar instead of starch in the root, are diastatically active in both leaf and root. The radish root is one of the most active tissues we have found, despite the fact that it contains only 5 per cent solid material, and yet no trace of starch could be found in the many samples examined. Other plants, like carrot, parsley and parsnip, lay up starch in the root which is diastatically active—in these cases more active than the leaf. The potato tuber is particularly rich in starch but rather poor in diastase.

In a very few cases tissues which were found to contain starch were so slightly active diastatically that they appear doubtful. The pulp of the marrow squash and the seed of the cantaloupe eventually carried digestion to the erythro stage, at which point it appeared to stop completely. Fresh mushroom tissues, after five days' digestion, showed no definite change of color. Curiously enough, these same tissues ground up in alcohol, washed with ether and dried, developed considerable diastatic activity. Did the treatment activate a proenzyme? If so, and the evidence of the single series of observations suggests this, why should the rapidly growing fresh tissues, rich in the glycogen-like carbohydrate of the mushroom, show no diastase? In the developing mushroom, which frequently grows several inches in a few hours, and in which the glycogen transfer must be unusually rapid, one would expect to find diastase in abundance if that enzyme is necessary for synthesis.

With the exception of these doubtful cases just mentioned the results of our series offer more favorable material to interpret from the standpoint of enzyme synthesis than any other investigation which we have made. The fact that practically all of the starch-storing tissues of plants are found to contain diastase, and that during the developmental stages as well as during sprouting, lends considerable support to the view that the enzyme is responsible for the synthesis of the polysaccharide from the sugar of the sap. It must not be lost sight of however that even these favorable results are by no means decisive; the data may be interpreted in a different way, and there is nothing in the experiments which is crucial. Furthermore if any of the doubtful cases cited should on further work prove to be actually negative, the value of the positive cases as favorable evidence for the theory would be entirely lost. At present, however, it appears to us that the strongest evidence for the theory is to be found in the relation of starch and diastase in the organs of plants. There are many tissues rich in diastase which never develop starch, but on the other hand there are no tissues which contain starch of which we can say that they are absolutely devoid of diastase. What the function of the diastase of tissues which never contain starch is, we have not even conjectured, but it seems quite possible, from the data, that those tissues which develop starch as a store of reserve material may synthesize the starch through the agency of diastase.



THE PROBLEM OF ENZYME SYNTHESIS. IV.

LACTASE OF THE MAMMARY GLAND.

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(Received for publication, November 11, 1912.)

In the preceding papers reporting investigations into the theory of enzyme syntheses in tissues, the results have been equivocal and difficult of interpretation. They have neither confirmed nor disproved the general proposition that enzymes assist in the synthetic production of compounds which they also hydrolyze. It is desirable therefore to secure some more crucial test of the theory. An organ or tissue whose chief function is synthetic and the products of whose synthesis are somewhat specific would make an ideal tissue to examine from this point of view. The active mammary gland is perhaps the best example of this sort of organ. It produces considerable amounts of protein, carbohydrate and fat, and the protein and carbohydrate are quite specific. Neither casein nor lactose are found elsewhere than in milk, and the lactose requires a specific enzyme to hydrolyze it. The number of tissues which contain this enzyme are moreover very limited. As Plimmer¹ has shown, intestinal mucosa and the pancreas of suckling mammals regularly contain the enzyme, while it is wanting in the adult pancreas and in most adult intestines. Its presence in significant amounts in the active mammary would therefore go far toward proving the theory under investigation.

As a test of the theory we have therefore proposed the presence or absence of lactase in the active mammary cell. The hydrolytic action of the enzyme is much more easily demonstrated than its synthetic action, so that the failure of Porcher² to get synthesis of lactose from a mixture of gland "Brei," dextrose and galactose,

¹ Plimmer: *Journ. of Physiol.*, xxxiv, p. 93; xxxv, p. 20, 1906.

² Porcher: *Arch. internat. Physiol.*, xxiii, p. 356, 1909.

is not conclusive. His results merely indicate that under the conditions of dilution, etc., which obtained in his experiments reversal did not take place. Dilution may have prevented Porcher from finding a synthetic effect, but dilution should only facilitate the hydrolytic reaction of the enzyme.

Active mammary glands were therefore obtained from animals suckling their young and at the height of lactation. Samples of the milk were secured in most instances and the presence of lactose demonstrated, as a preliminary precaution. The glands were ground to a fine "Brei" and diluted with a known proportion of water and were either allowed to autolyze over night under toluene or used immediately. The autolyzed and diluted "Brei" was strained free from connective tissue shreds and a known amount of the mixture added to a solution of Kahlbaum's C. P. lactose. A control digestion was checked at once by the addition of mercuric nitrate, prepared according to the method of Patein and Dufau,² or by boiling and then adding the mercuric nitrate. Other samples were allowed to digest from two to seven days under toluene at 37°. At the end of this period the proteins were removed by mercuric nitrate, the large excess of mercury precipitated in an aliquot of the protein-free filtrate by NaOH, and H₂S run in to precipitate the remainder. Excess of sulphide was then removed by CuSO₄ and the solution made up to a definite volume and filtered. Sugar was determined in aliquots of this filtrate by the Allihn gravimetric method. Another aliquot of the sugar solution was then completely hydrolyzed by boiling with H₂SO₄ for one hour, neutralized, made up to known volume and analyzed for sugar. Thus the reducing power of each solution was checked by its reducing power after hydrolysis—an extremely important point in the work, since it enables one to determine whether there has been destruction of either dextrose or lactose during the digestion period. The mere fact that the reducing power of the digest does not alter during a seven-day period is not of itself sufficient to prove the absence of lactase, since a concomitant destruction of sugar may have balanced the rate of hydrolysis. Nor would an increased reducing power of the solution alone be a proof of the hydrolysis of lactose, since liberation of a reducing substance in the

² Patein and Dufau: *Journ. de pharm. et de chim.*, xv, p. 221, 1902.

gland "Brei" itself is quite possible. The results of Plimmer may be subjected to valid criticism on this very ground, since increased reducing power of the digest was taken as proof of lactase, without determining also the amount of lactose remaining at the end of the digestion period. As a matter of fact we have found some indications of both destruction of dextrose and of the liberation of reducing disaccharides in certain experiments. The check has a further value. In the precipitation of the proteins by the mercuric nitrate method the character of the precipitate is frequently different in different digestion flasks. Presumably too there is a difference in the amount of sugar absorbed by such precipitates, so that several digestions made up with scrupulous attention to exact duplication of the amounts of sugar and "Brei" present, will frequently show appreciable differences in the amounts of sugar present in the final filtrates. Such differences must be assumed therefore in the interpretation of results as experimental errors inherent in the process, and variations within such limits cannot be considered evidence of hydrolysis. If lactase is present at all, however, a digestion of seven days should show changes far beyond these experimental errors, and give unequivocal evidence of hydrolysis.

In carrying out the details of this work we have followed the procedure described by Plimmer as closely as possible in order that our results might be comparable with his, and that some judgment might be made as to the amount of lactase present in the mammary gland compared with the tissues examined by him.

EXPERIMENT I. *October, 1911.* Cat with four kittens, toward the close of lactation but still nursing the kittens. The milk contained a reducing sugar. The glands, dissected free from fat and connective tissue, weighed 35 grams. They were reduced to a fine "Brei" with the addition of toluene water and allowed to incubate at 37° for twenty-four hours. The "Brei" was strained, the residue washed and the mixture made up to 250 cc. with toluene water. Four digests were set up:

- I. 50 cc. "Brei," 100 cc. 5 per cent lactose solution: incubated 24 hours.
- II. 50 cc. "Brei," 100 cc. 5 per cent lactose solution: incubated 48 hours.
- III. 50 cc. "Brei," 100 cc. 5 per cent lactose solution: incubated 72 hours.
- IV. 50 cc. "Brei," 100 cc. 5 per cent lactose solution: boiled at once as control.

At the end of each digestion period 10 cc. of mercuric nitrate solution were added, the mixture allowed to settle in the cold over night and filtered. One hundred and twenty cubic centimeters of the filtrate were made exactly neutral to litmus with concentrated NaOH and sufficient water added to make the total volume 125 cc. The precipitated mercuric hydroxide was then filtered, and H_2S passed into 100 cc. of the filtrate till the slight excess of mercury was removed. The excess of H_2S was precipitated by the addition of copper sulphate and the mixture made up to exactly 250 cc. and filtered. Sugar was determined in 25 cc. aliquots of this filtrate, which contains a very slight excess of the copper salt insufficient to exercise any appreciable effect upon the sugar determinations. The cuprous oxide was filtered on a Gooch, washed repeatedly with hot water, alcohol and ether, and dried at 105° for an hour. Duplicate results were obtained in each case.

Fifty cubic centimeters of the final filtrate were hydrolyzed with the addition of 2 cc. of concentrated sulphuric acid, boiled for one or more hours, neutralized and made up to 200 cc. Sugar estimations on this showed the total available reducing sugar and thus the undigested lactose could be determined.

The twenty-four and forty-eight-hour digests show a slight increase in reducing power, which is more than counterbalanced by a decreased total sugar. Our only explanation of this is the possibility of some slight bacterial action unchecked by toluene. The seventy-two-hour digest shows little deviation from the control.

TABLE I.

NO.	HOURS	CO_2O	AVERAGE	TOTAL AVAILABLE	RATIO
		<i>gram</i>			
1	0	0.4355			
		0.4354	0.4355	0.6178	1.418
2	24	0.4491			
		0.4470	0.4480	0.5704	1.273
3	48	0.4411			
		0.4405	0.4408	0.5832	1.323
4	72	0.4352			
		0.4345	0.4348	0.6000	1.379

EXPERIMENT II. *October, 1911.* Goat in active lactation, secreting about 400 cc. of milk per day. The milk contained

lactose. One hundred and sixty grams of gland tissue were secured, ground fine, diluted and autolyzed over night. Liver tissue from the same animal was made up in the same way as a check.

The results show no digestion of lactose. Where liver tissue is substituted for mammary there is a distinct loss of sugar.

TABLE II.

NO.	TIME	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	TISSUE
	<i>hours</i>	<i>per cent</i>				
1	0	14	0.4357	0.6180	1.419	Mammary.
2	96	14	0.4366	0.6200	1.420	Mammary.
3	0	28	0.4577	0.6252	1.366	Mammary.
4	96	28	0.5674*	0.7688	1.355	Mammary.
5	0	14	0.4715	0.6450	1.378	Liver.
6	96	14	0.4517	9.6152	1.362	Liver.

* A stronger lactose solution was used in this digestion by mistake; the ratio clearly shows however that no digestion has taken place.

EXPERIMENT III. *November, 1911.* Rabbit, twenty-four hours after dropping her litter. The glands were swollen and full of milk. Forty-five grams were ground fine, diluted to 250 cc. and autolyzed over night. The strained mixture was made up with lactose as in experiment I.

In Nos. 2 and 3 there is no evidence of a change of any kind; in 4, which was made distinctly alkaline to litmus with bicarbonate, there was a slight loss of sugar as shown by both the digestion and inverted sample.

TABLE III.

NO.	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		<i>per cent</i>				
1	0	18	0.3936	0.5360	1.362	
2	72	18	0.3939	0.5400	1.371	
3	168	18	0.3928	0.5398	1.374	
4	72	18	0.3751	0.5276	1.406	Alkaline with NaHCO ₃ .

EXPERIMENT IV. *December, 1911.* Goat in the height of lactation. The milk was shown to contain lactose. Two hundred

A number of objections may be urged at the outset. First: The method is not a quantitative one; the end point with iodine is not sharp and a considerable error is thereby introduced. Making all possible allowances for error in this direction does not however invalidate the general results—a given tissue is ten or a hundred times more active than another.

Second: Many tissues already contain starch in larger or smaller amounts, and the presence of this starch must inevitably alter the time required for digestion to the achromic point. This error is in many cases a serious one. In many others however the starch grains are so resistant to the diastase that filtering the samples taken for tests removes them, and the solution then represents the original starch solution added. We have found a number of tissues where the reaction of the starch grains in suspension persisted hours or even days after the soluble starch solution had all been converted into dextrans which gave no iodine color reaction.

Third: Very noticeable differences exist between the action of diastases of different origins. In some cases erythrodextrin is rapidly formed from the starch, and then persists for long periods of time. In others no erythrodextrin reaction develops; the solution remains blue-reacting to the end, and merely decreases in intensity to the vanishing point. In others the steps from starch to erythrodextrin to achroödextrin are well defined and follow each other in approximately the same time. This difference is possibly an indication of a complex of enzymes in what is called "diastase," one factor of which hydrolyzes starch, another erythrodextrin, etc. If, in a given tissue "Brei" the erythrodextrin-digesting enzyme is much more abundant than the starch-digesting, then the speed of the entire reaction will be expressive of the speed of that initial step of hydrolysis. The reddish color at the same time will never be appreciable, since the erythrodextrin will be hydrolyzed as rapidly as formed. In other cases, where the initial step is rapidly taken but the dextrinase is wanting, the mixture rapidly assumes the red-staining stage and then very slowly or never passes on to the achromic point.

Razor sections of the organs were stained with iodine, and where this reaction was negative or equivocal, samples of the "Brei" were extracted with boiling water and the extract tested for starch.

TABLE I.

SPECIES	TISSUE	DRY WEIGHT	DIGESTION TIME	STARCH CONTENT
		<i>per cent</i>	<i>hours</i>	
Green pea.....	leaf		0.70	None.
	pod and seeds	12.4	0.50	Small amount.
	seeds, medium	24.6	0.50	Medium.
	pod, medium	13.2	0.80	Small amt. dextrin.
	seeds, mature	80.0	1.00	Abundant.
Lima bean.....	seed, medium	23.2	6.00	Abundant.
	pod, medium		5.00	Small amt. dextrin.
	leaf	15.4	0.50	Small amt. dextrin.
Kidney.....	seed, medium	27.6	5.50	Medium.
	pod, medium	14.8	5.50	Abundant dextrin.
	leaf	18.0	0.25	Small amt. dextrin.
Kohlrabi.....	root		1.50	None.
	leaf		1.50	None.
Onion.....	root, young	13.4	?	None.
	leaf, young	10.6	48.00	None.
Bayberry.....	leaf		72.00	None.
	berry, mature		72.00	None.
Radish.....	root, mature	5.6	0.70	None.
	leaf, mature	8.6	0.70	None.
Carrot.....	root, medium	10.8	2.50	Abundant.
	leaf, medium		22.50	None.
	root, young		?	None.
Red beet.....	leaf, young		1.25	None.
	root, mature		14.00	None.
	leaf, mature		6.00	None.
Mangel.....	root, mature		?	None.
	leaf, mature		7.00	None.
	root, medium		18.00	Some dextrin.
Potato.....	tuber, medium		17.00	Very abundant.
	leaf, medium.		0.75	None.
	seed, medium		7.50	Abundant.
Corn.....	husk, medium		6.00	None.
	seed, mature		6.50	Abundant.
	cob, mature		6.50	Small amount.
	seed, young		5.00	Medium amount.
	cob, young		6.50	Small amount.
	husk, young		2.00	None.
Marrow squash	seed, mature	25.2	1.25	Medium.
	pulp, mature		?	Abundant.
	pulp, young		48.00	Abundant.
Cantaloupe ...	leaf, mature		0.50	None.
	pulp, mature		72.00	None.
	seed, mature		?	Small amount.

In the table the relative amounts of starch found are indicated. In general it is a fact that leaves contain starch. In some it is found always, in others only while photosynthesis is in rapid progress. The fact that our plant tissues were usually secured early in the morning, before photosynthesis had gone on to any large extent, is probably the explanation of our failure to find starch in many of the green parts of plants. During the night, the starch, in such parts as the leaves, usually disappears. It is believed to be converted into sugar and removed by the sap to the organs of storage.

About one hundred tissues were examined in this way. The results are very divergent. A few of the more striking figures are given in the table below. The publication of the rest would not add materially to the solution of the present problem.

The above typical results, together with the mass of data unpublished, shows the following conclusions are warranted:

First: Diastase is as a rule most abundant in the leaves, where starch is never stored permanently, but where it may be found in small amounts during photosynthesis. Leaves of different species exhibit very marked differences in their diastase content. The leaves of the bayberry, onion and leek contain so little of the enzyme as to be doubtful. On the other hand the leaves of the legumes were among the most active tissues examined. It is of interest to note that the leaves of the legumes were usually found to contain considerable starch or a dextrin-like compound, though there were several exceptions noted.

Second: No general deductions can be drawn relating the diastatic activity of a tissue with its starch-storing function. We find plants like the beets and mangels, which store no starch in the root but instead store sugar, having a highly active leaf and no diastase in the root. Other plants like the radish and rutabaga and kohlrabi, which also store sugar instead of starch in the root, are diastatically active in both leaf and root. The radish root is one of the most active tissues we have found, despite the fact that it contains only 5 per cent solid material, and yet no trace of starch could be found in the many samples examined. Other plants, like carrot, parsley and parsnip, lay up starch in the root which is diastatically active—in these cases more active than the leaf. The potato tuber is particularly rich in starch but rather poor in diastase.

In a very few cases tissues which were found to contain starch were so slightly active diastatically that they appear doubtful. The pulp of the marrow squash and the seed of the cantaloupe eventually carried digestion to the erythro stage, at which point it appeared to stop completely. Fresh mushroom tissues, after five days' digestion, showed no definite change of color. Curiously enough, these same tissues ground up in alcohol, washed with ether and dried, developed considerable diastatic activity. Did the treatment activate a proenzyme? If so, and the evidence of the single series of observations suggests this, why should the rapidly growing fresh tissues, rich in the glycogen-like carbohydrate of the mushroom, show no diastase? In the developing mushroom, which frequently grows several inches in a few hours, and in which the glycogen transfer must be unusually rapid, one would expect to find diastase in abundance if that enzyme is necessary for synthesis.

With the exception of these doubtful cases just mentioned the results of our series offer more favorable material to interpret from the standpoint of enzyme synthesis than any other investigation which we have made. The fact that practically all of the starch-storing tissues of plants are found to contain diastase, and that during the developmental stages as well as during sprouting, lends considerable support to the view that the enzyme is responsible for the synthesis of the polysaccharide from the sugar of the sap. It must not be lost sight of however that even these favorable results are by no means decisive; the data may be interpreted in a different way, and there is nothing in the experiments which is crucial. Furthermore if any of the doubtful cases cited should on further work prove to be actually negative, the value of the positive cases as favorable evidence for the theory would be entirely lost. At present, however, it appears to us that the strongest evidence for the theory is to be found in the relation of starch and diastase in the organs of plants. There are many tissues rich in diastase which never develop starch, but on the other hand there are no tissues which contain starch of which we can say that they are absolutely devoid of diastase. What the function of the diastase of tissues which never contain starch is, we have not even conjectured, but it seems quite possible, from the data, that those tissues which develop starch as a store of reserve material may synthesize the starch through the agency of diastase.



THE PROBLEM OF ENZYME SYNTHESIS. IV.

LACTASE OF THE MAMMARY GLAND.

By H. C. BRADLEY.

(From the Department of Physiology, University of Wisconsin.)

(Received for publication, November 11, 1912.)

In the preceding papers reporting investigations into the theory of enzyme syntheses in tissues, the results have been equivocal and difficult of interpretation. They have neither confirmed nor disproved the general proposition that enzymes assist in the synthetic production of compounds which they also hydrolyze. It is desirable therefore to secure some more crucial test of the theory. An organ or tissue whose chief function is synthetic and the products of whose synthesis are somewhat specific would make an ideal tissue to examine from this point of view. The active mammary gland is perhaps the best example of this sort of organ. It produces considerable amounts of protein, carbohydrate and fat, and the protein and carbohydrate are quite specific. Neither casein nor lactose are found elsewhere than in milk, and the lactose requires a specific enzyme to hydrolyze it. The number of tissues which contain this enzyme are moreover very limited. As Plimmer¹ has shown, intestinal mucosa and the pancreas of suckling mammals regularly contain the enzyme, while it is wanting in the adult pancreas and in most adult intestines. Its presence in significant amounts in the active mammary would therefore go far toward proving the theory under investigation.

As a test of the theory we have therefore proposed the presence or absence of lactase in the active mammary cell. The hydrolytic action of the enzyme is much more easily demonstrated than its synthetic action, so that the failure of Porcher² to get synthesis of lactose from a mixture of gland "Brei," dextrose and galactose,

¹ Plimmer: *Journ. of Physiol.*, xxxiv, p. 93; xxxv, p. 20, 1906.

² Porcher: *Arch. internat. Physiol.*, xxiii, p. 356, 1909.

is not conclusive. His results merely indicate that under the conditions of dilution, etc., which obtained in his experiments reversal did not take place. Dilution may have prevented Porcher from finding a synthetic effect, but dilution should only facilitate the hydrolytic reaction of the enzyme.

Active mammary glands were therefore obtained from animals suckling their young and at the height of lactation. Samples of the milk were secured in most instances and the presence of lactose demonstrated, as a preliminary precaution. The glands were ground to a fine "Brei" and diluted with a known proportion of water and were either allowed to autolyze over night under toluene or used immediately. The autolyzed and diluted "Brei" was strained free from connective tissue shreds and a known amount of the mixture added to a solution of Kahlbaum's C. P. lactose. A control digestion was checked at once by the addition of mercuric nitrate, prepared according to the method of Patein and Dufau,² or by boiling and then adding the mercuric nitrate. Other samples were allowed to digest from two to seven days under toluene at 37°. At the end of this period the proteins were removed by mercuric nitrate, the large excess of mercury precipitated in an aliquot of the protein-free filtrate by NaOH, and H₂S run in to precipitate the remainder. Excess of sulphide was then removed by CuSO₄ and the solution made up to a definite volume and filtered. Sugar was determined in aliquots of this filtrate by the Allihn gravimetric method. Another aliquot of the sugar solution was then completely hydrolyzed by boiling with H₂SO₄ for one hour, neutralized, made up to known volume and analyzed for sugar. Thus the reducing power of each solution was checked by its reducing power after hydrolysis—an extremely important point in the work, since it enables one to determine whether there has been destruction of either dextrose or lactose during the digestion period. The mere fact that the reducing power of the digest does not alter during a seven-day period is not of itself sufficient to prove the absence of lactase, since a concomitant destruction of sugar may have balanced the rate of hydrolysis. Nor would an increased reducing power of the solution alone be a proof of the hydrolysis of lactose, since liberation of a reducing substance in the

² Patein and Dufau: *Journ. de pharm. et de chim.*, xv, p. 221, 1902.

gland "Brei" itself is quite possible. The results of Plimmer may be subjected to valid criticism on this very ground, since increased reducing power of the digest was taken as proof of lactase, without determining also the amount of lactose remaining at the end of the digestion period. As a matter of fact we have found some indications of both destruction of dextrose and of the liberation of reducing disaccharides in certain experiments. The check has a further value. In the precipitation of the proteins by the mercuric nitrate method the character of the precipitate is frequently different in different digestion flasks. Presumably too there is a difference in the amount of sugar absorbed by such precipitates, so that several digestions made up with scrupulous attention to exact duplication of the amounts of sugar and "Brei" present, will frequently show appreciable differences in the amounts of sugar present in the final filtrates. Such differences must be assumed therefore in the interpretation of results as experimental errors inherent in the process, and variations within such limits cannot be considered evidence of hydrolysis. If lactase is present at all, however, a digestion of seven days should show changes far beyond these experimental errors, and give unequivocal evidence of hydrolysis.

In carrying out the details of this work we have followed the procedure described by Plimmer as closely as possible in order that our results might be comparable with his, and that some judgment might be made as to the amount of lactase present in the mammary gland compared with the tissues examined by him.

EXPERIMENT I. *October, 1911.* Cat with four kittens, toward the close of lactation but still nursing the kittens. The milk contained a reducing sugar. The glands, dissected free from fat and connective tissue, weighed 35 grams. They were reduced to a fine "Brei" with the addition of toluene water and allowed to incubate at 37° for twenty-four hours. The "Brei" was strained, the residue washed and the mixture made up to 250 cc. with toluene water. Four digests were set up:

- I. 50 cc. "Brei," 100 cc. 5 per cent lactose solution: incubated 24 hours.
- II. 50 cc. "Brei," 100 cc. 5 per cent lactose solution: incubated 48 hours.
- III. 50 cc. "Brei," 100 cc. 5 per cent lactose solution: incubated 72 hours.
- IV. 50 cc. "Brei," 100 cc. 5 per cent lactose solution: boiled at once as control.

At the end of each digestion period 10 cc. of mercuric nitrate solution were added, the mixture allowed to settle in the cold over night and filtered. One hundred and twenty cubic centimeters of the filtrate were made exactly neutral to litmus with concentrated NaOH and sufficient water added to make the total volume 125 cc. The precipitated mercuric hydroxide was then filtered, and H_2S passed into 100 cc. of the filtrate till the slight excess of mercury was removed. The excess of H_2S was precipitated by the addition of copper sulphate and the mixture made up to exactly 250 cc. and filtered. Sugar was determined in 25 cc. aliquots of this filtrate, which contains a very slight excess of the copper salt insufficient to exercise any appreciable effect upon the sugar determinations. The cuprous oxide was filtered on a Gooch, washed repeatedly with hot water, alcohol and ether, and dried at 105° for an hour. Duplicate results were obtained in each case.

Fifty cubic centimeters of the final filtrate were hydrolyzed with the addition of 2 cc. of concentrated sulphuric acid, boiled for one or more hours, neutralized and made up to 200 cc. Sugar estimations on this showed the total available reducing sugar and thus the undigested lactose could be determined.

The twenty-four and forty-eight-hour digests show a slight increase in reducing power, which is more than counterbalanced by a decreased total sugar. Our only explanation of this is the possibility of some slight bacterial action unchecked by toluene. The seventy-two-hour digest shows little deviation from the control.

TABLE I.

NO.	HOURS	Cr_2O	AVERAGE	TOTAL AVAILABLE	RATIO
		grams			
1	0	0.4355			
		0.4354	0.4335	0.6178	1.418
2	24	0.4491			
		0.4470	0.4480	0.5704	1.273
3	48	0.4411			
		0.4405	0.4408	0.5832	1.323
4	72	0.4352			
		0.4345	0.4348	0.6000	1.379

EXPERIMENT II. October, 1911. Goat in active lactation, secreting about 400 cc. of milk per day. The milk contained

lactose. One hundred and sixty grams of gland tissue were secured, ground fine, diluted and autolyzed over night. Liver tissue from the same animal was made up in the same way as a check.

The results show no digestion of lactose. Where liver tissue is substituted for mammary there is a distinct loss of sugar.

TABLE II.

NO.	TIME	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	TISSUE
	<i>hours</i>	<i>per cent</i>				
1	0	14	0.4357	0.6180	1.419	Mammary.
2	96	14	0.4366	0.6200	1.420	Mammary.
3	0	28	0.4577	0.6252	1.366	Mammary.
4	96	28	0.5674*	0.7688	1.355	Mammary.
5	0	14	0.4715	0.6450	1.378	Liver.
6	96	14	0.4517	0.6152	1.362	Liver.

*A stronger lactose solution was used in this digestion by mistake; the ratio clearly shows however that no digestion has taken place.

EXPERIMENT III. *November, 1911.* Rabbit, twenty-four hours after dropping her litter. The glands were swollen and full of milk. Forty-five grams were ground fine, diluted to 250 cc. and autolyzed over night. The strained mixture was made up with lactose as in experiment I.

In Nos. 2 and 3 there is no evidence of a change of any kind; in 4, which was made distinctly alkaline to litmus with bicarbonate, there was a slight loss of sugar as shown by both the digestion and inverted sample.

TABLE III.

NO.	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		<i>per cent</i>				
1	0	18	0.3936	0.5360	1.362	
2	72	18	0.3939	0.5400	1.371	
3	168	18	0.3928	0.5398	1.374	
4	72	18	0.3751	0.5276	1.406	Alkaline with NaHCO ₃ .

EXPERIMENT IV. *December, 1911.* Goat in the height of lactation. The milk was shown to contain lactose. Two hundred

and ninety-five grams of gland tissue were obtained. A 10 per cent lactose solution was used.

In this case there seems to have been a progressive liberation of sugar, more striking in the digestion to which blood had been added. The necessity of making a determination of the total available carbohydrate is clearly brought out in this instance, since an examination of the amount of reduction produced by the digestions alone would seem to indicate hydrolysis. The ratio shows however that such has not been the case.

TABLE IV.

NO.	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		<i>per cent</i>				
1	0	35	0.5450	0.8072	1.481	
2	120	35	0.5762	0.8144	1.413	
3	120	35	0.5918	0.8560	1.447	Blood present.

EXPERIMENT V. *February, 1912.* Cow in the height of lactation, killed as a demonstration of the tuberculin reaction. No macroscopic lesions were found in the mammary gland, which weighed over three kilos. Five per cent lactose solutions were used in the digestion mixtures.

Two and three were set up as duplicate digestions; the differences between them indicate the extent of divergence between such duplicates which must be taken into account in interpreting results. In five, the "Brei" was allowed to autolyze for seven days, the lactose solution then added and action stopped at once with mercuric nitrate. The series seems to show a small increase in the total sugar content as well as a larger increase in the reduction of the mixture before hydrolysis. That this is not a digestion of lactose itself is shown by Nos. 4 and 5. Comparing Nos. 1 and 4 there is some indication of increased disaccharide content. It may be suggested that a mother substance of lactose is present in the gland cells, as was thought by Porcher, from which lactose is split off during the digestion period. Whatever the explanation, the fact that a disaccharide is produced during digestion from the gland cells, is of itself in direct opposition to the theory that lactase is responsible for the lactose of the milk.

TABLE V.

NO.	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		<i>per cent</i>				
1	0	35	0.4059	0.5738	1.414	Lactose added at end of diges- tion.
2	72	35	0.4033	0.5752	1.426	
3	72	35	0.4184	0.5804	1.387	
4	168	35	0.4220	0.6078	1.441	
5	168	35	0.4252	0.5800	1.364	

EXPERIMENT VI. The mammary of the same cow was kept frozen hard at a temperature of about -20° for several days. While thus frozen it was possible to produce an exceedingly fine gland-snow with an ice shave. Another series of digestions were set up with this material, varying the reaction by the addition of bicarbonate sufficient to make the mixtures slightly alkaline to litmus.

The presence of alkali in the mixture leads to destruction of the sugar, though toluene was abundant in the mixtures and they were well shaken every twelve hours to insure saturation throughout the mixtures. There was no evidence of putrefactive changes in any of the mixtures, though bacterial count was not made. It is possible that in alkaline reacting "Breis" bacterial action was not wholly checked by the toluene, though this explanation of the destruction of sugar seems unlikely. In all the digests acidity is developed presumably through the action of lipase on the butter fats present.

TABLE VI.

NO.	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		<i>per cent</i>				
1	0	35	0.3995	0.5772	1.445	Alkaline.
2	96	35	0.3541	0.5052	1.427	
3	168	35	0.2735	0.3982	1.456	
4	168	35	0.3731	0.5220	1.402	
5	168	35	0.3201	0.4648	1.452	

DISCUSSION.

It is quite evident that under the conditions of these experiments no appreciable hydrolysis occurs. Therefore no lactase is present, for our only criterion of an enzyme is the demonstration of its activity. It is possible that a proenzyme normally present has not been activated, though conditions have been varied in the hope of producing such activation. Blood of the animal has been added to the mixtures, to determine whether it might not carry some kinase to the gland. Digestions have proceeded under neutral, slightly acid and slightly alkaline conditions. Sugar has been added at once to the freshly prepared gland-brei, and to "Brei" which has autolyzed for twenty-four hours. The result has been negative in each case. In certain digestion mixtures there has been an increased reduction observed as digestion continued, which might have been mistaken for hydrolysis of lactose were it not for the control hydrolysis by acid. In such cases the evidence points to the formation of a reducing disaccharide rather than to the conversion of disaccharide to monosaccharides of greater total reducing power. It seems safe to assume that this disaccharide is lactose, developed from some mother substance already laid up in the gland cells at the time of death. This mother substance may be analogous to glycogen, though the negative results of Porcher and other investigators makes this improbable; or it may be some more complex compound like a protein-lactose conjugation. The very fact however that autolyzing mammary tissue develops a reducing disaccharide, presumably lactose, is itself strong evidence that lactose is not built up by the enzyme lactase in the active gland.

There is further evidence that the production of lactose is not through the immediate agency of lactase. The secretion of milk is a violently eruptive process in which cells are ruptured and their contents poured out into the ducts. Following the manipulation of the teat by the suckling young, this eruptive secretion is set in motion by a reflex nervous mechanism. During the periods between suckling, the milk precursors are stored in the mammary cells as is shown by the swelling of the whole gland. If lactase were present at the moment of secretion it would certainly be swept out of the cells along with the milk constituents and would appear

in the secretion. If it were present in the milk, dextrose and galactose would gradually appear in place of the lactose. But dextrose is not found in normal fresh milk, nor on standing a few hours or days does it appear. At the moment of secretion therefore we must conclude that the enzyme is not present. It is possible that mammary lactase is an enzyme which is destroyed almost as rapidly as formed, but such a hypothesis is wholly without foundation in data thus far collected. Lactase from other sources is apparently quite resistant.

So far as this experiment goes, then, it gives no confirmation of the theory of enzyme syntheses. On the contrary it is so thoroughly negative in its results and so crucial in character that it must cast some doubt upon the general hypothesis. It appears justifiable to conclude that lactose of the milk is not synthesized through the agency of the enzyme lactase.



THE ACTION OF YEAST ON YEAST NUCLEIC ACID.

BY SAMUEL AMBERG AND WALTER JONES.

(From the Department of Physiological Chemistry, Johns Hopkins University.)

(Received for publication, November 12, 1912.)

Upon a former occasion we showed that rabbit's serum brings about a depression of the optical rotation of yeast nucleic acid without setting free either phosphoric acid or purine bases and apparently without causing any deep-seated chemical alteration of the substance. On the other hand thymus nucleic acid was found unchanged in any way by rabbit's serum since not even an alteration of the optical properties could be observed.¹ In the meantime Levene and Jacobs² have been led to conclude that the two nucleic acids are quite dissimilar in their chemical constitution so that differences in their biological conduct are to be expected.

We have recently had occasion to note a very marked difference in the behavior of these two nucleic acids towards various preparations of yeast, having uniformly found that yeast nucleic acid disappears, while under the same conditions thymus nucleic acid persists.

Experiments were made with a number of preparations, especially brewer's yeast, compressed yeast and yeast powder which we prepared by the method of Lebedew;³ but the results were always the same in that thymus nucleic acid was unchanged and could be precipitated by the addition of sulphuric acid while yeast nucleic acid disappeared and its decomposition products could be found. Thus a 1.5 per cent solution of yeast nucleic acid was completely decomposed by compressed yeast extract in three days while a solution of thymus nucleic acid under the same conditions was apparently unchanged after digestion for nineteen days. In an experiment with extract of yeast powder

¹ Amberg and Jones: this *Journal*, x, p. 81.

² *Ibid.*, xii, p. 411.

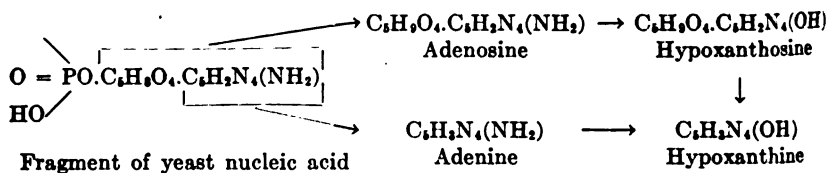
³ *Zeitschr. f. physiol. Chem.*, lxxiii, p. 447.

a 4 per cent solution of yeast nucleic acid was completely decomposed in three days while a 0.5 per cent solution of thymus nucleic acid was unaltered at the end of a month.

The decomposition products of yeast nucleic acid obtained under the conditions stated are not without interest as will be seen from the following general summary of results.

	COMPRESSED YEAST	YEAST POWDER
Autolysis.....	Adenine and xanthine	Adenine and guanine
With addition of yeast nucleic acid	Adenine and guanine	Adenine and guanosine

It will be observed first, that where one is dealing with an uninjured yeast preparation (as compressed yeast) and where the amount of nucleic acid to be decomposed is comparatively small (as in autolysis) the adenine group is set free but not deaminized while the guanine group is both liberated and deaminized. This may be defined as the normal condition for yeast and is in accord with the older findings that yeast contains guanase but no adenase.⁴ But the occurrence of adenine and the failure of hypoxanthine among the products of auto-digestion of yeast are matters that are unique in nucleolysis. There are two paths along which hypoxanthine may be formed from yeast nucleic acid: the one by liberation and deaminization of adenine, a decomposition to which the presence of adenase is indispensable; the other, by the initial formation of adenosine which is transformed successively into hypoxanthosine and hypoxanthine, a succession of reactions which can occur in the absence of adenase.



Most animal tissues do not contain any adenase so that one of these paths of hypoxanthine formation is out of question;

⁴ Straughn and Jones: this *Journal*, vi, p. 245.

nevertheless all such tissues bring about a formation of hypoxanthine from nucleic acid⁶ and evidently along the second path under consideration. But the yeast is characterized by its inability to bring about a deaminization of the adenine group (whether this be free or in combination) and therefore cannot produce hypoxanthine from any of its purine precursors. It will be seen in the experimental part which follows that, no matter how the experiment is arranged, adenine is *always* formed, hypoxanthine, *never*.

In the second place, it will be noted, that yeast powder which was prepared by manipulation of yeast has lost the power of converting guanine into xanthine, which is to say, the ferment guanase, normally present in fresh yeast, has been destroyed. We have had occasion to notice the easy destruction of guanase in other connections. For instance, a dog's liver was perfused with large quantities of distilled water until all hemoglobin had been removed. An extract of this bloodless liver was found free from guanase although a second dog's liver perfused with normal saline was found to possess the ferment unimpaired. In both instances hypoxanthine was found but no adenine.

Finally, it may be observed that the end products of the action of yeast on yeast nucleic acid depend upon the initial amount of nucleic acid to be decomposed—a curious phenomenon which was produced both by fresh yeast and by yeast powder. Compressed yeast proceeds with a small amount of nucleic acid as far as xanthine but when the initial amount of nucleic acid is considerably increased, guanine is found as the end product. Similarly, yeast powder can liberate guanine from a small amount of nucleic acid, while a larger amount of nucleic acid proceeds only as far as guanosine.

The isolation of purine derivatives and phosphoric acid from digested yeast preparations is much more difficult than in the case when one is dealing with glandular extracts; so that severe losses often occur in the estimation of the products of digestion. Nevertheless the results obtained are sufficient to establish the conclusions that have been stated.

⁶ Amberg and Jones: *Zeitschr. f. physiol. Chem.*, lxxiii, p. 407.

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EXPERIMENTAL PART.

EXPERIMENT I. 300 grams compressed yeast; 1000 cc. water; digestion, 19 days.

	Without hydrolysis. gram
685 cc. for purine bases.	
Adenine picrate.....	0.661
Xanthine.....	0.117
100 cc. for phosphorus.	
Mg.NH ₄ .PO ₄ .6H ₂ O.....	0.660

EXPERIMENT II. 275 grams compressed yeast; 920 cc. water; 15 grams yeast nucleic acid; digestion, 19 days.

	Without hydrolysis. gram	After hydrolysis. gram
400 cc. for purine bases.		
Adenine picrate.....	1.115	1.237
Guanine.....	0.135	0.310
Xanthine.....	0.0	0.060
Hypoxanthine.....	0.0	0.0
50 cc. for phosphorus.		
Mg.NH ₄ .PO ₄ .6H ₂ O.....	0.592	0.549

The increase in the amount of purine bases by hydrolysis may have been due to the presence of their precursors in the digested product, but it is likely that hydrolysis removed substances affecting the solubility of silver and copper compounds used in the isolation.

EXPERIMENT III. 100 grams compressed yeast; 300 cc. water; 375 mgm. guanine chloride; digestion, 19 days.

	Without hydrolysis. gram
250 cc. for purine bases.	
Adenine picrate.....	0.111
Guanine.....	0.0
Xanthine (loss through accident).....	0.075
Hypoxanthine.....	0.0

EXPERIMENT IV. 100 grams compressed yeast; 300 cc. water; digestion, 8 days.

	After hydrolysis. gram
Adenine picrate.....	0.365
Guanine.....	0.064
Xanthine.....	0.083
Hypoxanthine.....	0.0

EXPERIMENT V. 100 grams compressed yeast; 300 cc. water; 300 mgm. guanine chloride: digestion, 8 days.

	After hydrolysis. gram
Adenine picrate.....	0.217
Guanine.....	0.064
Xanthine.....	0.315
Hypoxanthine.....	0.0

EXPERIMENT VI. 150 grams yeast powder; 750 cc. water: digestion, 10 days.

	Without hydrolysis. gram	After hydrolysis. gram
200 cc. for purine bases.		
Adenine picrate.....	0.141	0.360
Guanine.....	trace.	0.189
Xanthine.....	0.0	trace.
Hypoxanthine.....	0.0	0.0
20 cc. for phosphorus.		
Mg.NH ₄ .PH ₄ .6H ₂ O.....	0.347	0.370

EXPERIMENT VII. 150 grams yeast powder; 750 cc. water; 26.5 grams yeast nucleic acid: digestion, 10 days.

The product proved difficult to handle. After a number of attempts at filtration and centrifugation in which a large amount of the material was either lost or abandoned, a small quantity of perfectly transparent fluid was finally obtained, but what proportion of the entire material was contained in this fluid we are unable to state. The solution was treated by the method of Levene and Jacobs⁶ as follows. Neutral lead acetate was added to the boiling hot fluid and the filtrate from the lead precipitate thus formed was treated with additional lead acetate and ammonia. This second lead precipitate was decomposed with sulphuretted hydrogen, the filtrate from the lead sulphide evaporated under diminished pressure and the macrocrystalline substance which deposited from the concentrated fluid by cooling to zero was washed first with cold water and then with alcohol. After a second crystallization from 80 per cent alcohol the material was dried in a desiccator and weighed. The yield was 0.928 gram of guanosine. The most perfect crystals of the substance were obtained by treating a hot solution in water or 10 per cent alcohol, with enough acetone to produce a turbidity, and allowing it to cool. The substance consisted of perfectly white aggregations of fine needles with pearly lustre and could not be distinguished in any way from guanosine prepared by the neutral hydrolysis of yeast nucleic acid under pressure. It did not contain any phosphorus.

⁶ Levene and Jacobs: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 2474, 2703; Levene and La Forge: *ibid.*, xliii, p. 3150.

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0.1738 gram substance required 11.33 cc. of standard sulphuric acid (1 cc. = 0.00331 gram nitrogen).

	Calculated for $C_5H_4N_2O \cdot C_4H_5O_1 \cdot 2H_2O$:	Found:
N.....	21.94 per cent.	21.52 per cent.

500 mgm. of substance were hydrolyzed with 5 per cent sulphuric acid. On cooling, the solution deposited colorless transparent crystals of guanine sulphate, and a drop of the supernatant fluid gave a strong reduction with Fehling's fluid with production of red cuprous oxide (*d*-ribose). The crystals of guanine sulphate were gotten again in solution by heating and the guanine was precipitated with ammonia. 0.238 mgm. guanine was obtained.

Theoretical guanine from guanosine, 47.3 per cent; found, 47.6 per cent.

The guanine after weighing was converted into various derivatives including the characteristic chloride and was thus thoroughly identified.

The ammoniacal filtrate from guanine after removal of the ammonia by boiling produced no precipitate with picric acid. The substance under discussion is guanosine beyond doubt.

We go somewhat into detail concerning this matter because we believe no one has hitherto described the isolation and identification of guanosine formed by the action of ferments on yeast nucleic acid. Of course guanosine could not be formed from thymus nucleic acid. Recently, Schittenhelm, London and Wiener examined the products of the action of intestinal juice on thymus nucleic acid and report the more or less certain finding of guanylic acid, guanosine and adenosine.⁷ Levene and Jacobs⁸ undertake to clear up the apparent discrepancy in the following language "Schittenhelm, London and Wiener have thought that they obtained guanosine on the digestion of thymo-nucleic acid by intestinal juice. The substance isolated by them gave the orcin test and had the appearance of guanosine. However, thymo-nucleic acid contains no pentose and therefore cannot yield guanosine (guanine riboside). Undoubtedly the nucleic acid employed by them was contaminated with guanylic acid. This consideration does not vitiate in any way the conclusion of those writers concerning the mechanism of nucleolysis" As we are inclined to believe that the considerations of Levene and Jacobs do rather profoundly affect the conclusions of those writers, we may be permitted to ask if the *adenosine* obtained by Schittenhelm, London and Wiener also undoubtedly resulted from the guanylic acid with which their specimen was contaminated.

⁷ *Zeitschr. f. physiol. Chem.*, lxxii, p. 459.

⁸ *This Journal*, xii, p. 377.

ANIMAL CALORIMETRY.

SEVENTH PAPER.

THE METABOLISM OF A DWARF.

BY F. H. MCCRUDDEN AND GRAHAM LUSK.

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New York City.*)

(Received for publication, November 13, 1912.)

Rubner¹ made experiments upon an American dwarf calling himself "General Mite" who was twenty years old and weighed only 6.6 kilograms. This corresponds to the weight of an infant at the breast. The individual in question ate a mixed diet and behaved in general like an adult. He exhibited himself in public, as a professional freak, dancing and singing. He produced daily 531 calories or 80.5 per kilogram of body weight, which may be contrasted with 70.1 calories per kilogram of body weight in an infant at the breast weighing 5.4 kilograms as determined by Rubner and Heubner. Per square meter of surface the active dwarf produced 1231 calories whereas the more quiet child produced 1006. An infant given cow's milk produced 1143 calories per square meter of surface. Rubner found in these figures the proof that size and not age determined the intensity of the metabolism.

EXPERIMENTAL PART.

The individual upon whom the following experiments were performed was a patient living at the Rockefeller Hospital for Medical Research who was brought to this laboratory for introduction into the calorimeter. His condition was that described by C. A. Herter² as intestinal infantilism. His metabolism as indi-

¹ Rubner: *Biologische Gesetze*, Marburg, 1887, p. 10; *Beiträge zur Ernährung im Knabenalter*, 1902, p. 45.

² Herter: *On Infantilism from Chronic Intestinal Infections*, 1908.

Metabolism											
DATE	EXPERIMENT NO.	TIME	CO ₂	O ₂	R. Q.	H ₂ O	URINE N	NON-PROTEIN			
								CO ₂	O ₂	R. Q.	Protein
1911											
April 6	1	<i>p.m.</i>	<i>grams</i>	<i>grams</i>		<i>grams</i>	<i>gram</i>	<i>grams</i>	<i>grams</i>		
		1.34-2.34	12.02	11.34	0.77	26.08	0.211	10.05	9.56	0.76	5.35
		2.34-3.34	11.98	9.66	0.90	26.93	0.211	10.01	7.88	0.92	5.35
April 7	2	1.34-2.34	12.05	11.27	0.78	17.05	0.230	9.90	9.33	0.77	6.10
		2.34-3.34	11.52	9.87	0.85	18.74	0.230	9.37	7.93	0.86	6.10
April 8	3	<i>a.m.</i>									
		11.10-12.10	10.00	9.52	0.76	16.14	0.162	8.49	8.15	0.76	4.29
		12.10-1.10	9.90	8.97	0.82	16.88	0.162	8.39	7.60	0.80	4.29
April 21	4	11.02-12.02	12.67	11.10	0.83	18.94	0.177	11.02	9.60	0.84	4.60
		12.02-1.02	11.20	9.36	0.87	24.42	0.177	9.55	7.86	0.88	4.60
May 1	5	<i>noon</i>									
		12.00-1.00	11.96	10.26	0.85	28.91	0.179	10.29	8.75	0.85	4.74
		1.00-2.00	11.69	9.55	0.89	24.41	0.179	10.02	8.04	0.91	4.74

cated by analyses of his urine and feces has already been elsewhere reported by one of the writers.³ The individual J. P. was seventeen years old, 113.3 centimeters high and weighed 21.3 kilograms naked. This gave him a calculated surface area of 0.946 square meters. He was accustomed to laboratory procedures. Introduction of the electrical resistance rectal thermometer and the subsequent hours spent in the calorimeter were in no way disturbing to him. He lay on a mattress placed on the floor of the calorimeter, his head resting on a pillow. He was provided with illustrated periodicals which he usually read during the first hour of the

³ McCrudden: *Journ. of Exp. Med.*, xv, p. 107, 1911; McCrudden and Fales: *ibid.*, xv, p. 113, 1912.

C. J. P.

BODY TEMPERATURE				MORNING WEIGHT	BEHAVIOR	FOOD
Total Feeding	Start	End	Difference			
36.68	37.40	37.31	-0.09	21.3 kgm.	Asleep 12 min.	No breakfast. At 11.30 a.m. 20 grams rice made into a pudding with cream and sugar.
32.72		37.10	-0.21		Asleep or quiet.	
69.40						
	37.19	37.03	-0.16		Quiet and reading.	No breakfast. At 11.30 a.m. 123 grams tenderloin steak (= 4.2 grams N). Refused to eat more.
		36.92	-0.11		Quiet.	
	37.28	37.15	-0.13		Quiet.	No breakfast. No food.
		36.95	-0.20		Quiet.	
32.33	37.26	37.20	-0.06		Quiet and reading.	Breakfast at 6.30 a.m. One glass milk, toast and one-half an orange.
34.70		37.08	-0.12		Quiet.	
67.03						
33.98	36.91	36.47	-0.44		Awake.	Customary breakfast (no record).
30.87		36.31	-0.16		Awake and asleep.	
64.85						

experiment after which he fell asleep or remained perfectly quiet.

The analytical results were obtained at an environmental temperature of 26° to 27° and are presented in Table I.

The basal metabolism.

As in the case of the determination of the basal metabolism of Dog II, this experiment was made about eighteen hours after the ingestion of the last meal. The dwarf partook of no breakfast. He complained a little of weakness. He was quiet throughout the experiment, the results of which were as follows:

Experiment 3. Metabolism of J. P., eighteen hours after food ingestion.

TIME	URINE N	NON- PROTEIN R. Q.	CALORIES		
			Protein	Non- protein	Total calculated
<i>a.m.</i>					
11.10-12.10	0.162	0.76	4.29	27.09	31.38
12.10- 1.10	0.162	0.80	4.29	25.45	29.74
					61.12

These figures show an average basal metabolism of 30.56 calories per hour. Calculated for the day the total heat production would be 733 calories, or 29 per kilogram of body weight and 775 per square meter of body surface. This last figure corresponds to those representing the basal metabolisms of Dog I (weight = 13.8 kgm.) and Dog II (weight = 9.3 kgm.) which were 759 and 784 respectively. All these figures agree within 3 per cent and the results show the verity of Rubner's law of skin area but place the amount of heat eliminated at a lower level than Rubner's figures indicate. This is because the experiments were performed when food was absent from the intestine and a condition of complete rest and absence from thermal influences prevailed. Similar results may be calculated from Rubner's⁴ own observations (Experiments 39-40) on a well-trained quiet dog weighing 4.6 kilograms which, during the first and second days of fasting, was kept at an environmental temperature of 33°. The surface area of this dog may be calculated to be 0.3079 square meter and the heat production at 746 and 752 calories per square meter of surface on the first and second days of fasting.

It is apparent from this that the *basal metabolism* is a truer starting point for comparative studies as regards the law of skin area than are experiments which include mechanical work, thermal influences and the results of food ingestion.

From Benedict's monograph on Inanition,⁵ one may estimate the basal metabolism in man as calculated from a night period extending from 1.00 to 7.00 a.m. and beginning thirty hours after the last ingestion of food. The following table shows the basal

⁴ Rubner: *Die Gesetze des Energieverbrauchs*, 1902, p. 323.

⁵ Benedict: *Metabolism in Inanition*, Carnegie Institution of Washington, 1907, pp. 480-83.

metabolism of various individuals, founded on the heat production per square meter of surface calculated for twenty-four hours.

Table showing calories per square meter of surface in twenty-four hours based upon the minimal night metabolism from the thirtieth to thirty-sixth hours of fasting in man.

EXPERIMENT NUMBER	INDIVIDUAL	BODY WEIGHT	PER SQUARE METER OF BODY SURFACE	BEHAVIOR
		<i>kgm.</i>	<i>calories</i>	
59	B. F. D.	67.8	834.8	Good sleep till 6.35 a.m.
68	A. L. L.	72.9	809.5	Asleep.
69	A. L. L.	73.8	905.7	Not stated.
71	S. A. B.	58.2	771.8	Asleep.
73	S. A. B.	59.1	856.3	Asleep till 3.30 then awake every hour.
75	S. A. B.	59.5	858.2	Slept fairly well.
77	S. A. B.	61.6	893.8	Felt too warm all night.
79	H. E. S.	57.2	1015.	Got up at 2.24 a.m. thinking it morning. Sick at stomach when he got up.
80	C. R. Y.	69.3	940.4	No record.
81	A. H. M.	62.0	738.8	Slept well all night.
82	H. C. K.	71.5	886.2	Awoke early.
83	H. R. D.	55.6	954.4	Sound sleep. Awake once.
85	N. M. P.	67.6	935.1	Woke up twice. Pneumograph uncomfortable.
89	D. W.	79.1	865.6	Woke up at 3.45 a.m. Dozed after this.

Benedict on p. 56 states of the experiment on A. L. L. "fasting experiment made under conditions ideal for obtaining minimum muscular activity" and on p. 107 he describes subject S. A. B. as being of "highly nervous temperament."

In experiments 59, 68, 71, 81 and 83 the individuals slept soundly all night. In the last named experiment (No. 83), the heat production was exceptionally high, 954 calories per square meter of surface. In the other four experiments the values 835, 809, 772 and 739 are found to average 789 calories per square meter of surface. The variation amounts to the considerable figure of 13 per cent, but the average is equal to the heat production found in our experiments.

The total heat production of the dwarf, therefore, conforms to Rubner's general law of skin area, which is a fundamental law

of metabolism. Only in the case of infants has exception to this law been found. Howland⁶ has shown that the metabolism of sleeping infants eighteen hours after food ingestion reaches 1100 calories per square meter of surface, indicating a higher heat production in the youthful protoplasm than is present in the adult.

As regards the kind of energy yielding material oxidized by the dwarf, it is noted that 14 per cent of the energy of metabolism is derived from protein and 86 per cent from fat and carbohydrate, a proportion which is entirely normal.

The effect of food ingestion.

Several experiments were performed to note the effect of food ingestion. On one morning no breakfast was taken until 11.30 a.m. when a large tenderloin steak was given. Of this only the equivalent of 123 grams of fresh lean meat was eaten, with a nitrogen content of 4.2 grams. This small quantity hardly raised the nitrogen elimination in the urine and caused no pronounced effect upon metabolism. A summary of all these experiments is presented below.

Metabolism of J. P. after food ingestion.

EXP. NO.	TIME	URINE N	NON- PROTEIN R. Q.	CALORIES				FOOD
				Protein	Non-protein	Total calculated	Total found	
1	<i>p.m.</i>							
	1.34-2.34	0.211	0.76	5.59	31.79	37.38	36.68	Carbohydrate and fat at 11.30 a.m.
	2.34-3.34	0.211	0.92	5.59	27.32	32.91	32.72	
						70.29	69.40	
2	<i>p.m.</i>							
	1.34-2.34	0.230	0.77	6.10	31.18	37.28		Meat at 11.30 a.m.
	2.34-3.34	0.230	0.86	6.10	27.06	33.16		
						70.44		
4	<i>a.m.</i>							
	11.02-12.02	0.177	0.84	4.69	32.60	37.29	32.33	Breakfast at 6.30 a.m.
	12.02-1.02	0.177	0.88	4.69	26.95	31.64	34.70	
						68.93	67.03	
5	<i>noon</i>							
	12.00-1.00	0.179	0.85	4.74	29.76	34.50	33.98	Breakfast.
	1.00-2.00	0.179	0.91	4.74	27.79	32.53	30.87	
						67.03	64.85	

⁶ Howland; *Zeitschr. f. physiol. Chem.*, lxxiv, p. 1, 1911.

In the four experiments cited above the calories of the protein metabolism amount to 16, 17, 14 and 15 per cent of the total metabolism which indicates the presence of entirely normal conditions.

The results presented naturally fall into two divisions, (1) the first hours during which illustrated papers or a book were being read prior to complete relaxation or sleep and (2) the second hours during which the boy was quiet or asleep throughout the period.

During three experiments the calculated heat production during the first hour was 37.38, 37.28 and 37.29 calories per hour, an average of 37.33 calories. During the second more quiet hours the heat production fell to 32.91, 33.16, 31.64 and 32.53 calories, an average of 32.55. Comparing these figures with the value of the basal metabolism the following results are obtained.

	CALORIES	INCREASE	
		Calories	Per cent
Basal metabolism.....	30.56		
Metabolism resting in bed after food.....	32.55	+1.99	6.6
Metabolism reading papers in bed..	37.33	+4.78	14.7

The experiments therefore led to the conclusion that the influence of small quantities of ingested food is to increase the metabolism by 6.6 per cent, whereas muscular work attendant upon reading in recumbent position causes a still further increase of 14.7 per cent. The influence of very moderate muscular activity is shown pronouncedly.

Calculated on the basis of twenty-four hours the metabolism may be measured as follows:

	CALORIES IN 24 HOURS		
	Total	Per kilogram	Per square meter of surface
Basal metabolism.....	733	33	775
Metabolism, reading after food.....	896	42	947

The authors gratefully acknowledge the assistance of Dr. H. B. Williams and Mr. J. A. Riche in the performance of this work.

CONCLUSIONS.

A dwarf, suffering from infantilism, seventeen years old and weighing 21.3 kilograms had a basal metabolism of 775 calories per square meter of surface in twenty-four hours which may be contrasted with 759 and 784 in two dogs of different weights.

The basal metabolism was increased 6.6 per cent as a result of the ingestion of food, and this again was increased by 14.7 per cent when the boy was reading illustrated periodicals in bed.

The protein metabolism yielded the normal proportion of 15 per cent of the total calories of heat production.

Nothing abnormal could be detected in the metabolic processes of the individual, as determined from these calorimetric observations.

ON THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS.

VIII. GLOBIN.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, November 16, 1912.)

A. THE PREPARATION OF GLOBIN.¹

Globin was prepared from ox corpuscles by three different modifications of the method of Schulz² as follows:

A thick suspension of ox corpuscles was obtained from freshly defibrinated ox blood by centrifugalization, the volume of the suspension being about one-third that of the blood from which it was derived.

After pipetting off the supernatant serum the suspension was diluted to the original volume of the blood from which it was derived by the addition of $\frac{4}{5}$ NaCl solution, and the centrifugalization was repeated, the supernatant fluid being removed as before. This was repeated six times in order to free the corpuscles from adherent serum. After the last centrifugalization the corpuscle suspension was not diluted again with NaCl.

The thick suspension of corpuscles which was thus obtained was diluted to ten times its volume by the addition of distilled water, the corpuscles were thus "laked" and the contained haemoglobin was discharged into the water, forming a clear solution which was allowed to stand in tall glass vessels for twenty-four hours in order to permit the leucocytes to settle. The upper portion of the fluid was then decanted and employed, the lower portion of the fluid being rejected.

¹ I am indebted to Dr. Charles B. Bennett for assistance in some initial attempts to prepare this substance.

² Fr. N. Schulz: *Zeitschr. f. physiol. Chem.*, xxiv, p. 449, 1898.

It has been shown by Preyer,³ Schulz⁴ and others that the addition of a sufficient concentration of acid to a solution of haemoglobin results in its decomposition into haematin and a protein, globin. The former of those substances is readily soluble in acidified ether, hence this decomposition affords a means of preparing globin and separating it from haematin. Schulz points out, however, that the concentration of acid employed to bring about the decomposition of the haemoglobin is of great importance in determining the completeness or otherwise of the subsequent extraction of the haematin by ether, and my own experiments abundantly confirm this observation. If too small a quantity of acid be employed the haematin is only very incompletely removed from the aqueous layer by extraction with ether. If too large a quantity of acid be employed the globin is denatured and forms with the ether a species of gel leaving the supernatant aqueous layer clear and colorless but free from globin.⁵ I have in no instance been able to obtain a perfectly colorless aqueous layer which still contains globin in a form precipitable by ammonia.

Preliminary experiments with the solution of corpuscles described above showed that the addition of 0.25 equivalent of HCl per liter afforded the best results. The aqueous layer was still considerably colored, but 0.38 equivalent, while permitting almost all the color to be extracted from the aqueous layer, also caused the greater part of the globin to leave the aqueous layer.⁶ On the other hand, 0.13 equivalent left the aqueous layer, after extraction with ether, very deeply colored and the separation into two layers was also very incomplete.

Accordingly 2.5 liter portions of the corpuscle solution were placed in six-liter bottles, to the contents of each bottle were added 56 cc. of concentrated HCl (specific gravity 1.18) and the mixture was thoroughly shaken and allowed to stand at room

³ Preyer: *Die Blutkrystalle*, Jena, 1871.

⁴ Fr. N. Schulz: *loc. cit.*

⁵ That is, failing to yield a precipitate upon the addition of ammonia.

⁶ Dr. Charles B. Bennett finds that once the decomposition of the haemoglobin into globin and haematin hydrochloride has been brought about by hydrochloric acid, calcium chloride added to the aqueous layer has the same effect upon the partition of haematin and globin between the ether and water as hydrochloric acid itself.

temperature for one hour. The addition of the acid caused a flocculent precipitate to appear and the mixture turned dark brown. Two and a half liters of ether were then added to each bottle and the contents were shaken thoroughly until they attained a thick oily consistency. Rubber stoppers with two perforations were then fitted into the necks of the bottles. Through one perforation was inserted a long glass tube reaching to the bottom of the bottle, to act subsequently as an air inlet, and through the other was inserted a short tube, just reaching to the bottom of the stopper and provided with a rubber tube and pinchcock. The stoppers were then tied down and the bottles quickly inverted and allowed to stand at room temperature for twenty-four hours.

By the following day the contents of the bottles had separated into two layers, an upper, jelly-like, very deeply colored ether layer and a lower, still somewhat deeply colored aqueous layer measuring about 1.7 liters. The latter was drawn off through the shorter of the two tubes inserted through the stoppers.

An attempt was made to further decolorize this fluid by adding to it more acid and then repeating the extraction with ether, with the very peculiar result that the addition of more acid, far from favoring the extraction of haematin by the ether, was found to actually *diminish* the amount of haematin taken up by the ether. In fact the addition of 0.05 or 0.10 equivalent per liter of HCl and subsequent extraction with an equal volume of ether resulted in a very rapid separation into an almost colorless ether layer and a still highly colored water layer; yet, as the experiments cited above show, had this acid been added *before* the first extraction it would have led to a more complete extraction of haematin and, in addition, to the inclusion in the jelly-like ethereal layer of the greater part of the globin. It seems that the ethereal extract of the original solution of blood-corpuscles must contain some substance or substances which enhance the solubility of haematin hydrochloride in ether and that when these are removed, although de-emulsification of the ether solution mixture is accelerated by acid, the power of the ether to take up haematin from the water is diminished.

On shaking up the still dark-colored solution with ether *without* any further addition of acid, de-emulsification of the mixture was extremely slow. I accordingly adopted the device employed

by Schulz,⁷ namely, that of adding to the ether one-quarter of its volume of alcohol. This resulted in quick separation of the mixture, after shaking, into two layers, an upper, ether-alcohol layer which was deeply colored and a lower aqueous layer somewhat less colored than before.

To 2 liters of the watery layer derived from the first extraction were added 2 liters of ether and 500 cc. of alcohol, and the mixture was thoroughly shaken. After allowing it to stand for a short period the watery layer was drawn off and the extraction repeated twice. After the last extraction the watery layer had become a pale opaque brown and the ether layer was only slightly colored. The watery layer was now diluted to ten times its volume by the addition of distilled water, and 150 cc. of a 20 per cent solution of ammonia were added. A precipitate appeared which quickly redissolved but on adding a nearly equivalent amount of HCl it reappeared, thus confirming the finding of Schulz that globin is insoluble in dilute ammonia in the presence of a sufficiency of ammonium chloride. This precipitate was collected upon a hardened filter-paper, washed in large volumes of alcohol and ether⁸ and dried over H₂SO₄ at 36°. After twenty-four hours it was pulverized, passed through a fine sieve and returned to the incubator to dry over H₂SO₄ for one week. The yield from 2 liters of the aqueous layer obtained after the first extraction, corresponding to 3 liters of the original corpuscle solution or 300 cc. of the corpuscle suspension (= about 900 cc. of blood) was 4.2 grams.

Globin was thus obtained in the form of a light very friable greyish powder, readily soluble in dilute ($\frac{N}{10}$) acids and alkalies,

⁷ *Loc. cit.*

⁸ According to Schulz (*loc. cit.*), globin, after washing with alcohol and ether and drying, is much less soluble in dilute alkalies and acids than normal globin. I have not observed this. It is true that dry globin dissolves much more slowly than wet globin, but this is a perfectly general phenomenon with proteins (cf. T. Brailsford Robertson: *Die physikalische Chemie der Proteine*, Dresden, 1912, p. 75) and is due simply to the fact that time is consumed in the penetration of the dry sponge-like particles of dehydrated protein by the solvent. (Cf. T. Brailsford Robertson: *ibid.*, p. 249.) I find, however, that if dry globin be brought into contact with dry acidified alcohol containing 0.1 equivalent of HCl per liter (prepared by passing dry HCl gas into absolute alcohol) not only does it fail to dissolve but it is rendered insoluble in dilute aqueous solutions of acids.

yielding, at a concentration of 2 per cent, light brown solutions. This substance will be referred to as Preparation I.

I observed that on adding to the watery layer obtained from the first extraction four volumes of a mixture of equal parts by volume of alcohol and ether, a light-colored precipitate of globin resulted, leaving the fluid very deeply colored, although the addition of four volumes of alcohol alone did not produce a precipitate. Accordingly to 1-liter portions of the fluid derived from the first extraction I added 2 liters of ether and 2 liters of alcohol. After allowing the precipitate to settle, which it did with tolerable rapidity, and decanting the greater part of the supernatant fluid, it was collected on a hardened filter, thoroughly drained, washed twice with 1 liter of alcohol and twice with 1 liter of ether within an incubator at 36° over H_2SO_4 and then dried in the incubator for from eighteen to twenty-four hours; at the end of which time it was pulverized, sifted and then returned to the incubator to dry for a week.

Three 1-liter portions of the watery layer derived from the first extraction with ether were treated in this manner. The total yield from this fluid, corresponding to 4500 cc. of the original corpuscle solution, 450 cc. of the corpuscle suspension and about 1350 cc. of blood, was 37 grams.

Globin was thus obtained in the form of a coarse brown powder, readily soluble in dilute acids and alkalies, yielding, at a concentration of 2 per cent, solutions somewhat more highly colored than those yielded by the preparation previously described. This substance will be referred to as Preparation II.

The alcohol-ether precipitate of globin, prepared in the manner described above, is readily soluble in dilute HCl and precipitated from this solution by ammonia.

Accordingly the globin from 1-liter portions of the watery layer derived from the first extraction with ether was precipitated by four volumes of alcohol-ether mixture, the precipitate collected upon a hardened filter, washed with 1 liter of alcohol, drained and then scraped off the paper and dissolved in 1 liter of $\frac{N}{10}$ HCl . This solution was then diluted to 4 liters, and 20 per cent ammonia solution carefully added until a precipitate just appeared (11 cc.). One cubic centimeter more of the strong ammonia was then added and the dense flocculent precipitate collected upon a hardened

filter. It filtered rapidly, the filtrate being pale yellow. The precipitate was then washed in alcohol (1 liter), alcohol-ether mixture (1 liter) and ether (2 liters), the washings with alcohol-ether mixture and ether being conducted within an incubator at 36° over H_2SO_4 . After draining, the precipitate was allowed to dry for twenty-four hours and then pulverized and sifted and returned to the incubator to dry over H_2SO_4 for one week.

Two 1-liter portions of the water layer derived from the first extraction with ether were treated in this manner. The total yield from this fluid, corresponding to 3 liters of the original corpuscle solution, 300 cc. of the corpuscle suspension and about 900 cc. of blood, was 20.6 grams.

Globin was thus obtained in the form of a very pale saffron-colored, light, friable powder, readily soluble in dilute acids and alkalies, yielding at a concentration of 2 per cent, light brown solutions less colored than those yielded by either of the preparations previously described. This substance will be referred to as Preparation III.

Schulz thus describes the product which he obtained, starting with crystallized haemoglobin: "Ein gelbliches, sehr lockeres, nicht wesentlich hygroskopisches Pulver." It differs from the majority of proteins by its high content of carbon (= 54.97 per cent, Schulz). It is predominantly basic and is considered by Schulz to be a histone. According to Abderhalden⁹ the globin from horse blood yields, on cleavage, notable quantities of leucine (29 per cent) and histidine (10.96 per cent.)

B. THE DETERMINATIONS OF REFRACTIVITY.

Two per cent solutions were made up of each of the above preparations in $\frac{N}{10}$ KOH, and portions of these solutions were diluted to 1 per cent by the addition of $\frac{N}{10}$ KOH. Two and one per cent solutions of Preparation III in $\frac{N}{10}$ HCl were also prepared. The refractive indices of these solutions and of the solvents ($\frac{N}{10}$ KOH and $\frac{N}{10}$ HCl) were measured at 18°C, in a Pulfrich refractometer, using *d*-sodium flame as the source of light.

⁹ E. Fischer and E. Abderhalden: *Zeitschr. f. physiol. Chem.*, xxxvi, p. 268, 1902; E. Abderhalden: *ibid.*, xxxvii, p. 484, 1903; E. Abderhalden and L. Baumann: *ibid.*, li, p. 397, 1907.

The following were the results obtained: The values headed a are calculated from the formula $n - n_1 = a \times c$ where n is the refractive index of the solution, n_1 that of the solvent and c is the percentage of protein in the solution.

TABLE I.

SOLUTION	n = REFRACTIVE INDEX OF SOLUTION AT 18° C.	a
$\frac{N}{10}$ KOH.....	1.33465	
$\frac{N}{10}$ HCl.....	1.33442	
1 per cent Preparation I in $\frac{N}{10}$ KOH.	1.33623	0.00158 ± 0.00008
2 per cent Preparation I in $\frac{N}{10}$ KOH.	1.33791	0.00163 ± 0.00004
1 per cent Preparation II in $\frac{N}{10}$ KOH.	1.33607	0.00142 ± 0.00008
2 per cent Preparation II in $\frac{N}{10}$ KOH.	1.33751	0.00143 ± 0.00004
1 per cent Preparation III in $\frac{N}{10}$ KOH.	1.33631	0.00166 ± 0.00008
2 per cent Preparation III in $\frac{N}{10}$ KOH.	1.33799	0.00167 ± 0.00004
1 per cent Preparation III in $\frac{N}{10}$ HCl..	1.33615	0.00173 ± 0.00008
2 per cent Preparation III in $\frac{N}{10}$ HCl..	1.33783	0.00171 ± 0.00004

It will be observed that in each case, within the experimental error (due to a possible error of $\pm 1'$ in reading the angle of total reflection), the difference between the refractivity of the solutions and that of the solvent is directly proportional to the concentration of protein dissolved in the solution. The values of a for the various preparations, however, differ somewhat. In order to assign to each observation its due weight in determining the average values of a for each of these preparations it is necessary to add together the observed values of $n - n_1$ for the different solutions (2 per cent and 1 per cent) of each preparation and divide this sum by the sum of the concentrations employed ($= 3$). Proceeding in this way we obtain:

TABLE II.

SUBSTANCE	VALUE OF a
Preparation I dissolved in $\frac{N}{10}$ KOH.....	0.00161 ± 0.00005
Preparation II dissolved in $\frac{N}{10}$ KOH.....	0.00142 ± 0.00005
Preparation III dissolved in $\frac{N}{10}$ KOH.....	0.00167 ± 0.00005
Preparation III dissolved in $\frac{N}{10}$ HCl.....	0.00171 ± 0.00005

The values of a for Preparation III dissolved in $\frac{N}{10}$ KOH and in $\frac{N}{10}$ HCl are identical within the experimental error; their aver-

age is 0.00169 ± 0.00005 . The value of a for Preparation I is slightly lower, while that of a for Preparation II is very much lower. These values obviously correspond with the relative purities of the preparations. It may be assumed, since both of them are of a very light color, that Preparations I and III are both very nearly pure, and, as we see, their refractivities are nearly equal. Preparation III, however, having been precipitated twice by different methods is less likely to be contaminated by impurities than Preparation I. On the other hand it is evident from its dark color that Preparation II is impure, and we see that its refractivity departs widely from that of Preparation III or that of Preparation I.

It appears probable, therefore, that the average value of the refractivity per gram per 100 cc. of solution of Preparation III determined above is, within the experimental error of the estimation, the refractivity, per gram per 100 cc. of solvent, of pure globin.

SUMMARY.

Globin has been prepared from ox corpuscles by three different modifications of the method originally employed by Schulz.

The value of a (= change in the refractive index of the solvent due to the introduction of 1 per cent of the protein) for the purest preparation obtained, when dissolved in $\frac{N}{10}$ KOH or $\frac{N}{10}$ HCl, is 0.00169 ± 0.00005 .

Less pure preparations yielded lower values of a .

THE SULPHATIDE OF THE BRAIN.

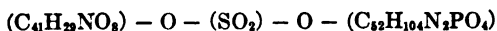
By P. A. LEVENE.

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New York.)

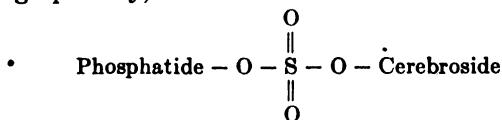
(Received for publication, November 21, 1912.)

There exists very little definite information regarding the nature of the sulphur-containing lipoids. The authors who are defending the individuality of protagon assume that both sulphuric and phosphoric acids enter into the structure of its molecule. On the other hand, Thudichum,¹ on the ground of theoretical considerations, was led to the belief that the sulphatide is a distinct lipoid having some properties in common with the phosphatides. Unfortunately he failed to separate the two substances. The ratio of sulphur to phosphorus in the purest sample analyzed by this author was 3:2, in other samples the ratio was 1:1.

In very recent years W. Koch² made the sulphatide the subject of a new investigation. Koch arrived at the conclusion that the sulphatide contained an equimolecular proportion of phosphoric and of sulphuric acids. The analytical data led him to formulate the structure of the substance in the following manner:



or, more graphically,



Thus the "phosphatide-cerebroside-sulphatide" of Koch contained all the elements that were supposed to be parts of protagon, and in a way the substance may have been regarded with some degree of justice as the purest protagon, although W. Koch militated against the existence of this complex.

¹ *A Treatise on the Chemical Constitution of the Brain*, London, 1884.

² *Zeitschr. f. physiol. Chem.*, lxx, p. 94, 1910.

In course of an investigation into the lipoids of the brain we found it possible to isolate the sulphur-containing lipid of the brain entirely free from phosphatides. In its physical appearance and in many of its properties the substance possessed a great resemblance to other lipoids and had the following composition:

0.1216 gram of the substance gave 0.2714 gram CO_2 and 0.1160 gram H_2O .

0.1216 gram of the substance gave 0.2706 gram CO_2 and 0.1150 gram H_2O .

0.2000 gram of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3.3 cc. of $\frac{N}{10}$ sulphuric acid.

0.4000 gram of the substance used for a sulphur estimation gave 0.0766 gram of barium sulphate.

0.4000 gram of the substance used for phosphorus estimation gave a negative result.

A comparison of the results of the analysis of the samples here described, and of those obtained by Thudichum and by Koch shows the following results:

	THUDICHUM	KOCH	LEVESE
C.....	47.70		60.90
H.....	7.54		10.67
N.....	1.14		2.31
P.....	3.94	1.80	0.00
S.....	6.19	1.91	2.66
O.....	33.49		23.46

The substance is dextrorotatory and melts at 210°C . (uncor.).

By the method employed for the preparation and purification of the sulphatide it was not possible to further purify the substance. Other methods will have to be devised for that purpose.

It must be remarked here that both Thudichum and Koch found that human brain offered a more suitable material for the preparation of their sulphatide. The substance described in this communication was isolated from beef brains.

Further work on the chemical structure of the substance is in progress.

A NOTE ON THE SUGAR TOLERANCE IN THE PIG.

BY A. J. CARLSON AND F. M. DRENNAN.

(From the Hull Physiological Laboratory of the University of Chicago.)

(Received for publication, November 27, 1912.)

According to Minkowski¹ almost complete pancreatectomy in the pig does not result in as severe diabetes as is the case in other mammals. The results obtained by Gould and Carlson² with almost complete pancreatectomy in two pigs seemed to confirm Minkowski's observation. In order to determine whether the pig's tissues possess unusual power to utilize sugars we made the following comparisons of the tolerance of two pigs before and after complete pancreatectomy. Three pigs of nearly the same size and age were selected. One of these was kept as control on the normal rate of growth.

Pig No. I.

Weight, 5 kgm.

1911, October 15-20. Dextrose dissolved in milk in quantities varying from 2.5 to 10 grams was given on empty stomach. *In every case sugar appeared in the urine within one hour after giving the sugar by mouth.* The total quantity of sugar excreted was not determined. The urine did not contain sugar when the pig was fed on milk unless dextrose was given by mouth.

October 22. Extirpation of the pancreas completed at 4 p.m. Urine was voided at 6, at 8 and at 10 p.m. All the samples contained sugar.

October 23. 170 cc. of urine passed in twenty-four hours, containing 10.20 grams of sugar.

October 24. 7 a.m. 80 cc. of urine, containing 5 grams of sugar. Animal died of peritonitis at 11 a.m. Post mortem showed complete removal of the pancreas.

¹ Minkowski: *Arch. f. exp. Path. u. Pharm.*, xxxi, p. 93, 1893.

² Gould and Carlson: *Amer. Journ. of Physiol.*, xxix, p. 174, 1911.

FIG No. II.

October 25-28. Milk diet. Quantity of urine during twenty-four hours on milk diet, 350-400 cc.

October 29. 10.30 a.m. Fresh sample of urine contained no sugar.

11.00 a.m. 5 grams of dextrose in 50 cc. of buttermilk given by mouth.

12.30 p.m. 50 cc. of urine (2 urinations) contained 0.531 gram of sugar.

1.45 p.m. 45 cc. of urine; no sugar.

2.30 p.m. 35 cc. of urine; no sugar.

October 30. 12 m. Fresh sample of urine contained no sugar. 5 grams of dextrose in 50 cc. of buttermilk given by mouth.

1.00 p.m. 40 cc. of urine contained 0.216 gram of sugar.

2.00 p.m. 15 cc. of urine contained 0.203 gram of sugar.

2.30 p.m. 20 cc. of urine contained 0.188 gram of sugar.

Total sugar = 0.607 gram.

October 31. 9.30 a.m. Sample of urine free from sugar.

10.00 a.m. 2.5 grams of dextrose in 50 cc. of buttermilk given by mouth.

10.20 a.m. Sample of urine contained no sugar.

11.00 a.m. 30 cc. of urine contained 0.133 gram of sugar.

12.00 m. 28 cc. of urine contained 0.250 gram of sugar.

12.30 p.m. Sample of urine free from sugar.

Total sugar = 0.383 gram.

During the time October 29-31 the pig was given no food except the quantities of dextrose and buttermilk shown above.

October 31. 1.00 p.m. The pig was allowed to eat as much as it desired of a mixture of bread and sweet milk.

2.00 p.m. 40 cc. of urine contained 0.68 gram of sugar.

November 5. 10.00 a.m. The pig was allowed to eat as much as it desired of cooked corn meal.

11.00 a.m. 50 cc. of urine contained 0.312 gram of sugar.

11.30 a.m. 50 cc. of urine contained 0.412 gram of sugar.

12.00 m. 35 cc. of urine contained 0.143 gram of sugar.

1.00 p.m. 100 cc. of urine contained slight traces of sugar.

Total sugar = 0.87+ gram.

November 12. After starving for two days the pig was allowed to eat as much as it desired of cracked corn. No sugar appeared in the urine.

November 21. Weight of pig, 8.2 kgm. Extirpation of the pancreas. During November 22-28 the daily ration consisted of 75 grams of bread in buttermilk.

November 22. 310 cc. of urine contained 23 grams of sugar.

November 23. 350 cc. of urine contained 58 grams of sugar.

November 24. 605 cc. of urine contained 92 grams of sugar.

November 25. 460 cc. of urine contained 59 grams of sugar.

November 26. 835 cc. of urine contained 114 grams of sugar.

November 27. 900 cc. of urine contained 128 grams of sugar.

November 28. 920 cc. of urine contained 126 grams of sugar.

From November 29 to January 8 the food consisted of bread and milk. The sugar excretion in the urine continued about the same as during the

week of November 22-28, but the pig was put in the metabolism cage each day only for the time required to secure one sample of urine.

January 8. The pig weighed 6.2 kgm., having lost 2 kgm. since November 21. When the pig was placed in the cage 450 cc. of urine were voided, in two hours, containing 52 grams of sugar. The pig was given nothing but buttermilk for forty-eight hours.

January 10. 270 cc. of urine voided in three hours, containing 30 grams of sugar. The bread and buttermilk ration was resumed.

January 12. 937 cc. of urine contained 113 grams of sugar. The feces contained no starch.

January 18. 1000 cc. of urine: Total sugar = 110 grams; total N = 11.58 grams.

January 21. After feeding 200 grams of bread in 500 cc. of buttermilk, 1300 cc. of urine: Total sugar = 151 grams; Total N = 14.1 grams.

No food was given January 21-24.

January 22. 830 cc. of urine: Total sugar = 71 grams; total N = 7.72 grams.

January 23. 1410 cc. of urine: Total sugar = 67.7 grams; total N = 12.0 grams. Weight of pig, 5.72 kgm.

The pig did not seem to recover well after the starvation period, January 21-24. The animal did not eat heartily and was lying down most of the time. On January 26, the animal refused food and water, so it was killed with ether. Weight, 5.60 kgm.

Post mortem findings. The pancreas was completely removed. The stomach was unusually dilated and contained 2280 cc. of fluid and ingested material. There was a large aneurism sac in the fundus (greater curvature) where the muscle layers were absent. The sac had been formed by a rupture of the muscle layers. The duodenum was greatly dilated and filled with fluid. The common bile duct was dilated to a diameter of 1 cm.

FIG No. III. CONTROL.

Several tests were made of the tolerance to dextrose *per os* with results identical to those reported in the cases of pig I and II. On November 21 (date of pancreatectomy of pig II, weighing 8.2 kgm.) pig III weighed 8 kgm. On January 27 this pig weighed 27 kgm. From November 21 to January 26 the diabetic pig lost weight at the rate of about 25 grams per day, while the normal pig gained at the rate of 300 grams per day. The actual loss of body materials by the diabetic pig was greater than these figures indicate, for more than a third of the weight of the pig at death represented fluid and ingested material in the enormously dilated stomach and duodenum.

1. While the results on pig II show that complete pancreatectomy is followed by the fatal diabetes typical of other species, we were impressed by the length of time the pig continued in fair condition and vigor. It is probable that the pig would have

lasted ten to twenty days longer if it had not been weakened by the three days' fast, January 21-23.

We have no explanation for the dilated condition of the stomach and the duodenum, unless it be essentially a mechanical effect, that is, an insatiate appetite resulting in ingestion of too great quantities of food for the weakened stomach walls.

2. The normal pig has a lower tolerance for dextrose, bread or cooked starch given by mouth than any species so far studied. When a normal pig weighing 8-10 kilos shows marked alimentary glycosuria when given 2.5 grams of dextrose, and that on an empty stomach, the lack of tolerance seems to border on the pathological. The lack of tolerance is, of course, due to failure of adjustment of the rate of absorption to the rate of fixation or storage of the sugar. We had planned to test the parenteral sugar tolerance in the pig, but that seemed useless on finding the slight tolerance *per os*.

This condition in the pig must lead to waste of calories in proportion to the quantity of bread or cooked starch fed.

3. It might be pointed out that the pig is a very convenient subject for physiological work involving frequent collections of the urine. The pig urinates on the average every thirty or forty-five minutes. The animal is almost as accommodating as if one had direct access to the ureters.

A NEW (COLORIMETRIC) METHOD FOR THE DETERMINATION OF URIC ACID IN BLOOD.

BY OTTO FOLIN AND W. DENIS.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, November 30, 1912.)

Notwithstanding the great physiological and clinical interest which for more than a hundred years has been attached to the "uric acid in blood" no suitable method for the determination of this uric acid has yet been discovered. The ammoniacal silver nitrate method of Salkowski and the cupric bisulphite method of Krüger for precipitating uric acid have indeed rendered valuable service, for by their means the presence of traces of uric acid in certain kinds of pathological blood has been positively established. Rough quantitative determinations of the uric acid in such blood have also been made on the basis of those methods though it is very doubtful how trustworthy have been the results.

Brugsch and Schittenhelm clearly recognize the doubtful character of their own quantitative uric acid determinations in blood on the basis of those methods,¹ and in fact regard the determination of as little as 1-3 mgm. of uric acid as practically impossible. They emphasize the value of the qualitative test for uric acid by the murexide test, a point which is well taken in view of the fact that the analytical methods are not sensitive enough to yield positive qualitative tests for the uric acid of normal blood.

An astounding feature of the procedure described by Brugsch and Schittenhelm for the detection and quantitative estimation of uric acid in blood is the recommendation² that the blood be treated with hot caustic potash solution (2-5 per cent) in connection with the preliminary precipitation of the proteins. Such a treatment with caustic alkalies is absolutely fatal since it would destroy anywhere from 30 per cent to all of the uric acid originally present.

In view of the well-known ease with which uric acid decomposes in alkaline solutions such a recommendation coming from Schittenhelm, who for

¹ *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 440, 1907; *Münch. med. Wochenschr.*, lix, p. 2377, 1912.

² *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 441, 1907.

years has been an active contributor in the field of the autolytic formation and destruction of uric acid and other purines, is certainly surprising.

As far back as 1901 Folin and Shaffer called attention to the great instability of uric acid in alkaline solutions and cited figures to show that over 30 per cent is lost when uric acid is heated on the water bath for forty-five minutes with 15 per cent sodium hydrate solutions.³ Folin and Shaffer traced the decomposition of the uric acid by titrations with potassium permanganate. We have now made another series of tests by the new colorimetric determination described in the last number of this *Journal* and from our results cite the following:

1. A 2 per cent NaOH solution containing 10 mgm. uric acid per 20 cc. lost 35 per cent by boiling three minutes.
2. A corresponding (2 per cent) Na_2CO_3 solution under the same conditions lost 15 per cent of the uric acid.
3. A 0.5 per cent NaOH solution in three minutes produced a loss of 18 per cent of the uric acid.
4. A 0.5 per cent Na_2CO_3 solution in three minutes destroyed 12 per cent of the uric acid.

We venture to suggest that the reason why Schittenhelm has overlooked the instability of uric acid is the fact reported by E. Fischer that uric acid solutions do not lose ammonia on being heated with normal caustic potash solutions.⁴ The fact that uric acid solutions do not lose ammonia is, however, by no means inconsistent with the fact that the uric acid is decomposed into something which no longer behaves like uric acid.

The crucial qualitative test for uric acid obtained from blood has always been the murexide reaction. The remarkable feature about the voluminous literature on the subject of uric acid in blood is the number of positive results reported for uric acid on the basis of this reaction. Most of these positive findings represent probably nothing but delusions. All know that since uric acid is abundant in the urine, traces of it must be in the blood and all have probably been more or less disposed to accept anything showing a reasonable resemblance to the murexide test as proving the presence of uric acid. But the murexide test is made in the same way as is the xanthoprotein reaction for proteins and the red color obtained in the latter is not so very different from the mixture of red and violet obtained in the murexide reaction. Moreover, the xanthoprotein reaction is given not only by the various protein materials in the blood but is also given by many phenol compounds, a number of which occur in blood. The murexide

³ *Zeitschr. f. physiol. Chem.*, xxxii, p. 555, 1901.

⁴ *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 3266, 1899.

reaction is therefore by no means reliable when applied to such unknown mixtures as have been obtained from the blood and this combination of circumstances is probably adequate to account for the many remarkable "uric acid" observations recorded in the literature.

There is no doubt, however, that by treating greatly concentrated mother liquor obtained from blood with ammoniacal silver nitrate or with copper bisulphite, traces of uric acid can be obtained. This is true not only for abnormal blood which is rich in uric acid but holds also for normal human blood, though the various methods heretofore in use have not been sensitive enough to reveal such traces of uric acid. Even in the case of gouty blood containing relatively large quantities of uric acid a great deal of blood (75 cc. or more)⁶ must be taken in order to obtain even a qualitative test. For quantitative determinations Brugsch and Schittenhelm use from 150 to 300 grams, *i.e.*, nearly 10 per cent of all the blood in the body.

The new method described in this paper is based on the highly delicate color reaction with our "uric acid reagent," a phosphotungstic acid solution by means of which one part of uric acid in one million parts of water gives a positive test. The color obtained from a quarter of a milligram of uric acid is quite adequate for a quantitative determination. Normal human blood, as we have discovered, contains about that much uric acid in from 15 to 25 cc. so that on the basis of such volumes we are now able to make quantitative determinations of the uric acid in blood.

For coagulating the proteins we use five volumes of boiling $\frac{N}{100}$ acetic acid solution (10 cc. of normal acetic acid to a liter of water). The blood is drawn into small, wide-mouth bottles previously weighed and containing a small amount (about 0.1 of a gram) of finely powdered potassium oxalate. From the subsequent weight of each bottle is obtained the weight of the blood. Five times this weight of $\frac{N}{100}$ acetic acid solution is transferred to an ordinary liter flask and heated to boiling. The oxalate blood is then poured into this boiling acetic acid solution and the heating is continued until the solution has again begun to boil. The mixture while still hot is filtered. The coagulated material on

⁶ Brugsch and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 442, 1907.

the filter paper is transferred back into the flask (by means of a small spoon or a spatula), about 200 cc. of boiling water⁶ are poured over it and it is allowed to stand a few (5) minutes. This mixture is then filtered through the same filter as was used for the first filtration. The filtrate in the receiving flask should be very nearly as clear as water and will be found to be so if the original blood was promptly shaken with the oxalate so that no clotting has taken place.

If clotting has occurred the coagulation and washing of the blood is a little more complicated. The clot leads to so much bumping in the boiling acetic acid solution that it is not practicable or safe to try to heat the mixture to boiling. The filtration is therefore made earlier. The partially coagulated clot is then broken up with a glass rod, transferred to a mortar and there ground into a paste in the presence of hot water. This suspension is then poured on the filter. The protein material on the filter is then washed as before with about 200 cc. of hot water. In this case the combined filtrates are, however, never colorless but more or less reddish. On being heated to boiling a second small coagulum will be obtained and the filtrate will then be practically as clear as water.

The combined filtrate and wash water containing the uric acid and other soluble materials is further acidified by the addition of 5 cc. of 50 per cent acetic acid and is evaporated over a free flame in a suitable porcelain dish⁷ to a very small volume (about 3 cc.). The liquid is then poured into an ordinary small centrifuge tube and the dish washed with two successive portions of 0.1 per cent lithium carbonate solution, using about 2 cc. for each rinsing, any solid material adherent to the sides of the evaporating dish being removed by rubbing with a rubber tipped stirring rod. To the liquid in the centrifuge tube, which at this stage should not be greater than 10 cc. in volume, is added 5 drops of 3 per cent silver

⁶ For this washing we use water, not $\frac{N}{100}$ acetic acid solution, because if the latter is used the coagulum will give off more or less of the blood pigment and the filtrates are less clear.

⁷ Deep (half globular) dishes 10 cm. in diameter and having a capacity of about 250 cc. are very good for this purpose. While free flames are the most convenient for the concentration of the uric acid solutions, care must of course be taken not to char the contents toward the end of the operation. Unless one is willing to watch it carefully at this stage it is safer to finish the concentration on the water bath.

lactate solution,⁸ 2 drops of magnesia mixture and a sufficient amount of strong ammonium hydrate (10–15 drops) to dissolve the silver chloride. The tube is centrifuged for two or three minutes, the supernatant liquid poured off and to the residue are added four or five drops of fresh saturated hydrogen sulphide water and one drop of concentrated hydrochloric acid. The tube is now placed for a period of five or ten minutes in a beaker of boiling water in order to remove the excess of hydrogen sulphide. Since hydrogen sulphide produces a blue color reaction with the phosphotungstic reagent it is of course important to remove every trace of this substance. To secure this a drop of 0.5 per cent lead acetate is added to the contents in the centrifuge tube as it is taken out of the hot water. Ordinarily very little or no lead sulphide (blackening) is produced, showing that all the hydrogen sulphide is gone as the lead acetate added is enough to give a heavy black precipitate. If such a precipitate is obtained it is safest to heat the tube in the water bath for another five minutes, and then add another drop of lead acetate solution. When all the hydrogen sulphide is thus removed the contents of the tube are centrifuged for one or two minutes. The supernatant liquid is then transferred by decantation as completely as possible to a small beaker and the inside of the tube washed with a stream of water from a wash bottle, care being taken to disturb as little as possible the solid residue at the bottom of the tube. The wash water (which should not exceed 5 cc. in volume) is then added to the liquid in the beaker and to this acid solution containing the uric acid is then added 2 cc. of the uric acid reagent⁹ and 10, 15 or 20 cc. of saturated sodium carbonate solution depending on whether the color obtained requires a final dilution to 25, 50 or 100 cc.

⁸ Silver nitrate is undesirable in this connection because traces of liberated nitric acid are not easily excluded and might destroy some of the uric acid. Folin and Macallum used silver sulphate to avoid this danger but the sulphate is very insoluble. The lactate is therefore the most serviceable silver solution that we have yet found for the precipitation of uric acid. Since several reagents are added drop by drop in this work it is very convenient to keep the solutions in so-called dropping bottles.

⁹ This reagent as stated in previous papers contains 100 grams of sodium tungstate and 80 cc. of phosphoric acid (85 per cent) per liter, all boiled together for one or two hours. See this *Journal*, xii, p. 239, 1912.

This direction with reference to the sodium carbonate to be used may seem somewhat vague to the casual reader but is really a simple matter in the actual work. The standard solution must already be made and from a rough comparison it is not difficult to determine whether the unknown is to be diluted to 25, 50 or 100 cc. in order to give somewhat near the same depth of color as that of the standard.

Three volumetric flasks representing 25, 50 and 100 cc., respectively, must be at hand, and the blue unknown solution is transferred to one of them and diluted with water up to the mark. The standard solution obtained from 1 mgm. of uric acid when treated with 2 cc. of uric acid reagent, 20 cc. of sodium carbonate and made up to 100 cc. should be made just before the addition of the sodium carbonate to the unknown. The latter sometimes needs to be filtered before being transferred to the colorimeter cylinders for the final color comparison. In all other respects the determination of uric acid in the blood is the same as the corresponding determination in urine.¹⁰

The calculation of the results obtained from the colorimetric readings is not difficult though it must not be forgotten that it is not practicable to take any particular quantity of blood for the work since the usual thing in clinical cases is that the amount of blood available is so small that one takes all that has been collected. The following formula gives the amount of uric acid per 100 cc. or more accurately per 100 grams of blood.

$$\frac{20 V}{R W} = \text{Milligrams of uric acid per 100 grams of blood.}$$

In this formula 20 represents the depth in millimeters of the standard solution used for a comparison and R represents the reading in millimeters of the unknown solution. V represents the volume (25, 50 or 100 cc.) to which the unknown is diluted and W represents the weight of blood taken for the determination.

We have made a great many uric acid determinations in human blood and in the blood of a number of different animals by the new method described above. Inasmuch as the data so obtained

¹⁰ Folin and Macallum: this *Journal*, xiii, p. 363, 1912. Blood, like urine, contains phenol compounds which give a blue color with the uric acid reagent. Because of the small amount of uric acid and relatively large amount of other materials in blood we have not attempted to determine the uric acid without any preliminary precipitation with silver.

possess considerable interest of their own, we prefer to make them the subject of a separate paper. Here we wish therefore to add only the determinations of uric acid previously added to blood, showing that by this method it is possible to determine the minute quantities occurring even in normal human blood.

Brugsch and Schittenhelm have recorded a few similar experiments¹¹ by which they tested the merits of the method which they employed in their quantitative work on the uric acid in blood. They, however, first dissolved the uric acid in boiling lithium hydroxide and then added from 100 to 200 mgm. of uric acid to each 100 grams of blood. Results obtained from such experiments have no bearing on the actual analysis of human blood containing at most only 8-10 mgm. per 100 cc.

In the experiments recorded below the uric acid solution (1 mgm. per cc.) was prepared by the help of lithium carbonate, as described in a previous paper,¹² and this solution was added in proportions of from 1 to 10 cc. to 100 cc. of sheep blood which contains no uric acid, *i.e.*, less than 0.05 mgm. per 100 cc.

VOLUME OF BLOOD TAKEN FOR ANALYSIS	URIC ACID ADDED	URIC ACID FOUND
cc.	mgm. per 100 cc.	mgm. per 100 cc.
50	0	less than 0.05
25	1	0.96
25	1	0.98
25	2	2.00
15	2	2.00
15	4	3.90
25	4	4.00
20	6	5.80
10	8	7.40
15	8	7.50
10	10	9.40
10	10	9.60

¹¹ *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 447, 1907.

¹² Folin and Macallum: *loc cit.*



A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF EPINEPHRINE.¹

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In an earlier paper Folin and Denis described the preparation of a phosphotungstic acid solution which proved extraordinarily sensitive as a reagent for uric acid and which also gave a similar (blue) color reaction with polyphenol compounds. One such phenol derivative is epinephrine. Solutions containing 1 part of epinephrine in 3,000,000 parts of water give an unmistakable reaction with this reagent. According to the seemingly careful comparative studies recently published by Borberg² none of the usual color reactions for epinephrine are capable of revealing the presence of this substance in dilutions greater than 1 part in 300,000 parts of water. Our reaction is therefore approximately ten times as sensitive as the best chemical tests hitherto proposed and in fact approaches in delicacy the remarkable physiological reactions which have heretofore been employed in testing for epinephrine. The elaboration of the reaction into a quantitative method for the determination of epinephrine in commercial suprarenal extracts as well as in "home made" extracts of suprarenal glands proved a comparatively easy undertaking.

Through the courtesy of Dr. Reid Hunt we were saved the laborious and indispensable task of preparing a sample of pure epinephrine. From him we obtained some of the ash-free epi-

¹ "The Council on Pharmacy and Chemistry of the American Medical Association has lately adopted the name Epinephrin for the active principle of the suprarenal glands in preference to using a 'protected' name and it is for this reason that we are using the word in this paper." See Abel: *Journ. of Pharmacol. and Exp. Therapeutics*, iii, p. 328, 1912.

² *Skand. Arch. f. Physiol.*, xxvii, p. 347, 1912.

nephrine purified by Taveau.³ By the help of this product we determined the chromophoric value of epinephrine in terms of another pure and easily obtainable substance (uric acid) which gives the same kind of a blue color with the phosphotungstic acid. Epinephrine gives almost exactly three times as much color as an equal weight of uric acid. Our determinations gave figures which varied between 2.90 and 2.95. As even our pure epinephrine probably is not as pure as Kahlbaum's uric acid with which we compared it, the ratio 1: 3 must be very nearly correct.

With this ratio once established it is no longer necessary to have on hand epinephrine solutions of known concentration in order to determine unknown epinephrine solutions. With uric acid, the phosphotungstic acid reagent and a high grade colorimeter available, it is now only a matter of a few minutes' work to assay a given solution of epinephrine. Using 10 cc. volumetric flasks, as little as three or four hundredths of a milligram of epinephrine, or even less, can be determined with a very satisfactory degree of accuracy.

To illustrate: A freshly opened bottle of "adrenalin" was used as a stock solution and from it one of us (Cannon) made a series of extremely dilute solutions. These together with the original solution were submitted to Folin and Denis for analysis. The following figures were obtained.

Original "a 1 per cent" solution:	Found: 0.11 per cent
A diluted to contain 25 parts per million:	Found: 26 parts
B diluted to contain 12.5 parts per million:	Found: 11 parts.
C diluted to contain 2.5 parts per million:	Found: 1.9 parts.

Having proved to the satisfaction of all of us that we were able to assay accurately pure epinephrine solutions, we next undertook to determine whether the crude extract obtained from suprarenal glands could be assayed colorimetrically for their epinephrine content. At first sight this seemed very dubious in view of the fact already established by Folin and Denis that blood contains enough uric acid to be estimated quantitatively, and contains besides phenol derivatives which, because of their reactivity with the uric acid reagent, necessitate a preliminary precipitation of the uric acid before it can be determined quantitatively. In this

³ Hygienic Laboratory Bulletin, iv, p. 23, 1909.

respect muscle, glands and other organs were found to behave in the same way. Fortunately, however, the blue color obtained by adding the uric acid reagent to extracts of blood and tissues other than the suprarenals is so very small compared with the color obtained from the latter as to be practically negligible. The suprarenals might of course contain some other specific products, besides epinephrine, capable of giving the same blue reaction as the latter. Although there is no reason why such should be the case it seemed desirable and indeed necessary to determine the point experimentally by checking up the values obtained colorimetrically by a standard physiological method.

Our extracts were made in the following manner: The weighed gland is thoroughly rubbed in a mortar with fine sand and tenth-normal hydrochloric acid. The mixture is then rinsed into an Erlenmeyer flask with more tenth-normal acid and water, using in all about 15 cc. of the acid for each 2 grams of gland and about three times as much water. The acid mixture is then heated to boiling. In the presence of so much hydrochloric acid there is no coagulation and the epinephrine is thoroughly dissolved. To the boiling mixture is then added a little 10 per cent sodium acetate solution (5 cc. for each 15 cc. of hydrochloric acid present) and the mixture is again heated to boiling. The albuminous material comes down promptly under this treatment. Instead of filtering and washing at this stage we transfer the whole mixture (except the sand) to a volumetric flask (capacity 100 cc. for each 2 grams of gland) and dilute up to the mark with water. The solution so obtained or as much of it as is needed can be filtered or centrifuged, giving finally a solution which is nearly or quite as clear as water. Ours were obtained by help of the centrifuge.

Notwithstanding the highly interesting and convincing character of Elliott's recent epinephrine determinations by the blood pressure method, we are inclined to think that from the standpoint of quantitative analysis his figures are probably not particularly exact, although the physiological reaction involved appears to be suprisingly regular. Epinephrine is extracted with considerable difficulty from coagulated material and it is rapidly destroyed by hot Ringer's solution. Elliott probably did not always get out all the epinephrine present and he certainly always destroyed some of it by using the more or less alkaline Ringer's solution.

The colorimetric determinations in the extracts described above were made at once as follows:

Five cubic centimeters of the clear extract were pipetted into a 100 cc. measuring flask and 1 cc. of a fresh uric acid solution containing 1 mgm. of uric acid was pipetted into another 100 cc. flask. To each flask were then added 2 cc. of the "uric acid reagent"⁴ and 20 cc. of saturated sodium carbonate solution. After standing two or three minutes the solutions were diluted to the 100 cc. mark, thoroughly shaken, and the color comparison was made in the usual manner in a Duboscq colorimeter with the uric acid standard set at 20 mm. On the basis of this color comparison the epinephrine was first calculated as though it were uric acid and the figure so obtained was divided by three, because epinephrine, as already indicated, produces three times as much color as an equal weight of uric acid.⁵ The figures obtained on the fresh suprarenal extracts are given in table I.

It being Saturday the solutions were transferred to the cold storage room. Two days later they were again assayed colorimetrically and their epinephrine content was then also determined by the blood pressure method.⁶

In the physiological assay the extracts of the glands were tested for their epinephrine content by the method recently described by

⁴ For the convenience of the recipients of reprints we give here again the composition of the "uric acid reagent." To 750 grams of water add 100 grams of sodium tungstate and 80 cc. of 85 per cent phosphoric acid. Boil gently (to prevent undue concentration) for one and one half to two hours and dilute to 1 liter. When the amount of adrenaline to be determined is so small as to require 10 cc., 25 cc. or 50 cc. flasks, the amount of reagent used should be correspondingly reduced (to avoid turbidity).

⁵ The description given above of the manner in which we made our colorimetric epinephrine determinations may also serve as a general description of the method, except that the volume of extract to be taken for a determination and the final volume to which it is to be diluted will depend on the amount of epinephrine present. The final blue color should be as deep as the standard obtained from 1 mgm. of uric acid, or even 10-20 per cent deeper. Weak colors are more difficult to compare because the blue from epinephrine is a little less bright, less transparent, than the blue produced with uric acid. Instead of uric acid the phosphotungstic acid reagent with an excess of epinephrine may be used as a standard. See Folin and Macallum: this *Journal*, xiii, p. 363, 1912.

⁶ By comparing the colorimetric values recorded in table I with the corresponding figures in table II it will be seen that though kept in a dark cold storage room having a temperature of 2-3°C. a considerable fraction of the epinephrine had decomposed in the course of two days.

TABLE I.
*Epinephrine content of different suprarenal glands.*⁷

KIND OF GLAND		WEIGHT OF GLAND	TOTAL EPINEPHRINE	EPINEPHRINE PER GRAM OF GLAND
		grams	mgm.	mgm.
Sheep (1)	1.....	2.25	6.66	2.96
	2.....	2.07	6.40	3.08
Sheep (2)	1.....	3.16	8.80	2.78
	2.....	2.99	7.80	2.60
Sheep (3)	1.....	2.66	6.66	2.50
	2.....	2.66	6.40	2.41
Lamb	1.....	1.17	3.00	2.56
	2.....	1.02	3.00	2.94
Cattle.....		11.69	46.00	3.96
Young cat	1.....	0.164	0.20	1.22
	2.....	0.164	0.25	1.52
Rabbit	1.....	0.239	0.76	3.18
	2.....	0.264	0.86	3.25
Dog	1.....	0.641	1.50	2.33
	2.....	0.620	1.27	2.04
Monkey	1.....	0.327	0.66	2.01
	2.....	0.360	0.86	2.39
Calf*	1.....	2.26	7.60	3.36
	2.....	2.08	7.32	3.52
Calf*	1.....	3.07	10.66	3.47
	2.....	2.65	8.82	3.33

* These glands were from "vaccine calves" and were obtained from Dr. Theobald Smith. The animals were killed by means of a blow on the head and immediate bleeding; their suprarenals like those of the slaughter-house animals therefore probably contain a normal amount of epinephrine.

Elliott.⁸ A cat was quickly etherized; a tracheal cannula was immediately inserted, and the brain then pithe'd. Artificial respiration without ether was at once begun and was continued through the experiment. Cannulas were fastened into the common carotid artery and into the external jugular vein. The jugular cannula was placed low in the neck so as to avoid as far as possible any ineffectiveness of the injected fluid through stasis in

⁷ The epinephrine determinations in the suprarenals of the cat, rabbit, dog and monkey were not made at the same time as the determinations on the slaughter-house animals, and the figures obtained from them probably do not represent the normal values because those animals had been used for other experiments before being killed.

⁸ Elliott: *Journ. of Physiol.*, xliv, p. 376, 1912.

the vessel. The medulla and upper portion of the spinal cord were next destroyed by a wire passed into the spinal canal through the orbit and brain case. Finally the carotid cannula was connected with a mercury manometer. The blood pressure now registered at a maintained level of about 40 mm. of mercury.

The adrenalin (Parke, Davis and Company's) used as a standard was 1 cc. of the original (1 : 1000) solution in 40 cc. of distilled water. Thus 1 cc. of the standard contained 0.025 mgm. An injection of 0.1 cc. of this solution, *i.e.*, 0.0025 mgm., would cause a marked rise of blood pressure. By repeated injections of the same dose the first record of the pressure change could be duplicated with remarkable exactness. Elliott's statement that the largely denervated circulatory system will respond with almost the accuracy of a chemical balance to any submaximal dose of adrenalin we were able to confirm.

The extracts were usually diluted 1 : 10 or 1 : 25 immediately before being tested. Each was placed in a burette connected with the jugular cannula, which was also filled with the extracts. At first 0.5 cc. of the extract was permitted to run slowly into the vein. If this amount gave a satisfactory submaximal rise of pressure, no further record was made. If necessary 1 cc. was injected.

By previous injections of various amounts of the standard solution, the approximate amount required to produce a rise of blood pressure equal to that caused by the extract could be judged. With the writing point of the manometer tracing the same zero line as before the estimated amount of the standard solution was now introduced into the vein. If the curve rose to the same height as previously the adrenalin content of the injected amounts of standard and extract was regarded as the same, and could be readily calculated. If the curve caused by injecting the standard was slightly lower than that caused by injecting the extract, a larger amount of the standard was given. And if this in turn resulted in a curve slightly higher than that of the extract, the intermediary value of the standard was taken as corresponding to that of the extract.

The results of the determinations made by the physiological process and by the colorimetric method are given in the following table.

TABLE II.

Epinephrine as determined (A) colorimetrically (B) by its effect on the blood pressure.

KIND OF SUPRARENAL GLAND		WEIGHT OF GLAND	TOTAL EPINEPHRINE BY COLORIMETRIC METHOD	EPINEPHRINE BY ELLIOTT'S BLOOD PRESSURE METHOD
		grams	mgm.	mgm.
Sheep (1)	{ 1.....	2.25	5.2	
	{ 2.....	3.07	4.6	5.0
Sheep (2)	{ 1.....	3.16	6.1	6.2
	{ 2.....	2.99	5.6	5.6
Sheep (3)	{ 1.....	2.66	5.2	5.0
	{ 2.....	2.66	5.0	5.0
Lamb	{ 1.....	1.17	2.2	2.3
	{ 2.....	1.02	2.2	2.5
Cattle.....		11.69	35.0	37.5

It should be stated in connection with the above figures that Cannon, who made the physiological test, did not know the values obtained by the colorimetric test, nor did Folin and Denis know the values obtained by the blood pressure process until all the figures were ready for comparison.

ADDENDUM.

It is worthy of note that incidental experiments have proved that the method here described is sufficiently delicate, when applied to blood taken from the adrenal veins, to give a stronger color reaction after stimulation of the splanchnic nerves than before. Thus to the various physiological evidences that splanchnic stimulation excites secretion of the adrenal glands is added chemical evidence.



NEPHELOMETRY IN THE STUDY OF PROTEASES AND NUCLEASES.

FIRST PAPER.

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(Received for publication, December 1, 1912.)

INTRODUCTION.

The author in studying ferments, required a method which would reveal, quickly and accurately, any change in the activity of the ferment during the course of an investigation. The following method, which is based on the use of a nephelometer, introduced into analytical chemistry by Richards,¹ seems to meet those requirements.

The following four requisites can be given for a good method of estimating proteases.²

1. The substance used for a substrate should be a protein.
2. The protein should be soluble in the solution of the ferment, in distinction to a reaction between phases.
3. Any and all unchanged protein should be completely precipitable by a convenient reagent.
4. This undigested protein should be determinable, accurately and quickly.

A suitable protein for peptic digestion is edestin,³ owing to its solubility in weak hydrochloric acid solution. Casein⁴ is well adapted for tryptic digestion, owing to its solubility in weak alkali

¹ T. W. Richards: *Zeitschr. f. anorg. Chem.*, viii, p. 269, 1895; Richards and Wells: *Amer. Chem. Journ.*, xxxi, p. 235, 1904; Richards: *ibid.*, xxxv, p. 510, 1906.

² The subject of suitable units for proteases will be discussed in the near future.

³ Fuld and Levison: *Biochem. Zeitschr.*, xi, p. 473, 1907.

⁴ Gross: *Arch. f. exp. Path.*, lviii, p. 157, 1907.

solution, and yeast nucleic acid in dilute solutions is similarly adapted for the study of nucleases.

Edestin⁵ is easily and quantitatively precipitated as edestan from its acid solution by 12 per cent sodium chloride solution. Casein can be completely thrown down by a weak acetic acid solution and other precipitants, while yeast nucleic acid can be rendered wholly insoluble by a slight excess of hydrochloric acid.

By using the specially devised nephelometer (easily made from the Duboscq colorimeter) described below, one can determine the amount of any undigested substance which is precipitated in the form of a suspension by these reagents, and thus follow the digestion of edestin with pepsin, of casein with erepsin and trypsin, etc., and yeast nucleic acid with nucleases quantitatively, with an error of less than 2 per cent. As a study of precipitants for casein and for yeast nucleic acid is still in progress the results on edestin only will be given in this paper.

METHOD.

(a) *Description of the instrument and its construction.*

As the nephelometer described by Richards yields its best results only on taking a large number of readings (from ten to twenty),⁶ and since for ferment work so much time for readings is objectionable, an improvement was highly desirable.

A similar instrument of greater accuracy, one that yields reliable results with a few readings comparable to those obtained with a Duboscq colorimeter, would greatly enhance the value of the nephelometer for physiological chemists. No doubt the Richards instrument sufficed for the purpose for which Richards designed it and therefore efforts to improve it were heretofore superfluous. Since, however, the instrument is not desired now to yield a correction to some other analytical process, but to form the basis of the analytical process itself and to yield all the figures of the determination, greater accuracy is required.

⁵ T. B. Osborne: *Journ. Amer. Chem. Soc.*, xxiv, p. 28, 1902.

⁶ As stated in a private communication to the author, the Richards instrument rarely departs more than 4 per cent from the true value on a single reading; the mean of these readings is within 2 per cent; but to attain accuracy within 0.5 per cent, many readings are needful.

Without going into the various considerations connected with the improvement of the nephelometer, it is sufficient to say that the optical workmanship of the Duboscq colorimeter proved to be an excellent basis on which to build a nephelometer, and the following therefore represents an attempt to obtain greater accuracy.⁷

The change from colorimeter to nephelometer, and *vice versa*, can be made in a few minutes with the simple, easily made additions described below. As most laboratories have a Duboscq colorimeter, an additional useful instrument can be produced in this way with little expense.

The essential differences between the new and the older nephelometers are as follows:

1. In the new instrument, a plunger changes the height of liquid under observation.
2. A shutter supplements the action of the plunger in cutting off the amount of light.
3. The new nephelometer eliminates three possible sources of error: the meniscus, the indirect reflection of light from suspended matter in the lower part of the tubes, and the reflection of light from one tube to another.
4. The dark shade reduces any error due to the reflection of light on the eyepiece.
5. The new instrument is more adaptable to daylight.
6. Less liquid is needed for a test, 6 cc. being the maximum amount needed.
7. The instrument is inclined, so as to make observations more convenient, and to prevent air bubbles from forming when the plungers are first introduced into the liquid.

The accompanying drawing, for which I am indebted to Mr. Sugiura, shows clearly enough the arrangements without much further description.

The tubes containing the liquid can be made from soft glass having a bore of 15.2-0.3 mm. by anybody having a little skill in glass blowing.

The nephelometer shown here was made by the author, without any special tools or skill, from small packing-box boards.

⁷ Judging only from the readings with the new nephelometer, greater accuracy was obtained but as both instruments were not compared by the same person under similar circumstances, this statement needs further confirmation.

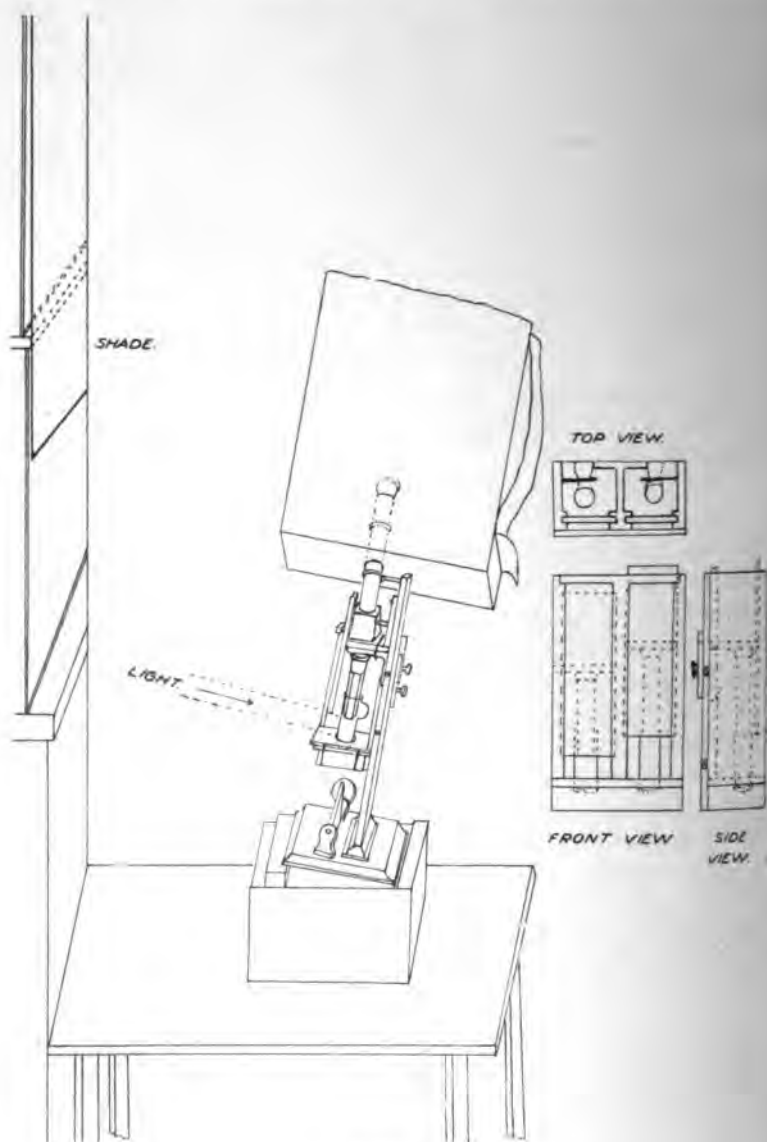


FIGURE 1.

The essential points are:

1. The sides of the glass plungers are given several coats of black asphaltum paint, leaving the circular ends clean, with sharp and well-defined edges. This change can be made permanent, as it does not interfere with the use of the instrument as a colorimeter.

2. A removable wooden partition, fastened to a wooden bottom plate as shown in the drawing, is fitted to the instrument. This partition serves, not only as a guide for the two shutters, but prevents reflection of light from one tube to another.

3. The shutters which are held to the plunger brackets by a spring clamp, are made of the same material, and serve to eliminate stray reflections.

4. The tube holder, which is fastened below the metal base, by means of two rubber bands, contains two holes which receive the bottoms of the tubes.

5. The eyepiece is inclosed in a wooden or paper box, with an opening sufficiently large to admit a man's head.

This eyepiece, as well as all other wooden additions, is covered with black asphaltum paint.

(b) *General considerations.*

As has been mentioned the instrument can be used with daylight, but for accurate work this light varies and is not always sufficiently constant. Reliable results are obtained with a 100 watt "Mazda" lamp,³ placed, well-screened, about one-half meter from the nephelometer.

To use the instrument for quantitative work, it must be carefully standardized with solutions of known strength, keeping the instrument under the exact lighting arrangements as in actual work. The amount of light observed in the eyepiece, as pointed out by Richards and by Wells is not proportional to the weight of the precipitate under observation, but seems to be dependent to some extent on the condition of the precipitate, the concentration or amount of substance per cubic centimeter and the height and thickness of the liquid.

If a standard solution is always used in the same way, *i.e.*, the same concentration and the same height of the liquid in the nephelometer, then the readings obtained with "unknown" solutions, plotted against the ratios of the solution, will follow with considerable accuracy, a definite and regular curve as may be seen in

³ I am indebted to Mr. W. F. Brady, Vice-President, New York Edison Company, for placing at my disposal a generous supply of different size tungsten lamps.

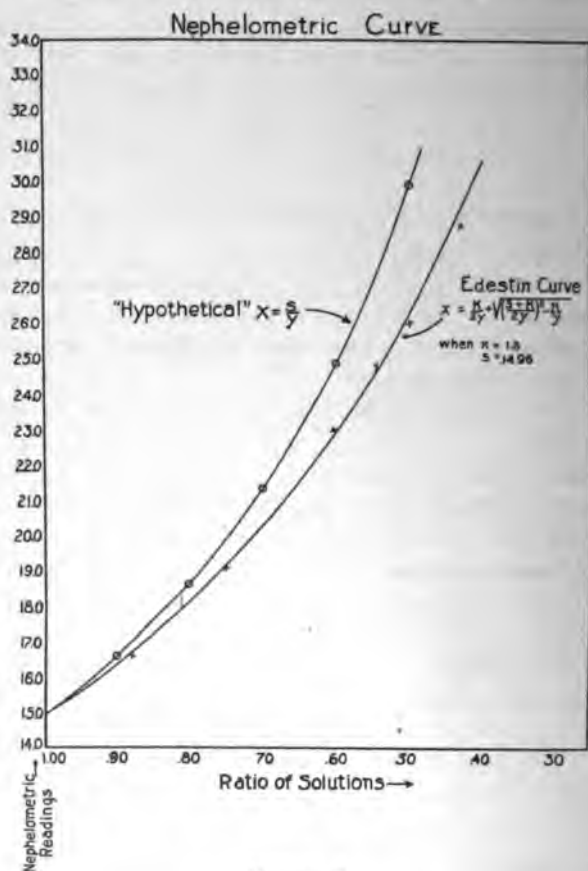


FIGURE 2.

figure 2. This curve, as may also be seen, is considerably lower than the "hypothetical" curve where the readings are inversely proportional to the concentration of the solutions. Richards and Wells assumed that when solutions of the same height gave the same amount of light, the ratios of the solutions were unity, and that around this ratio the "hypothetical" curve was correct. This is shown to be true by the curves in the chart within 1.5 per cent when the ratios are within 10 per cent of unity. It was therefore necessary, for Richards and Wells, to adjust the volume of their solutions, until the ratios were within this limit.

In the following work with edestin a scheme is presented which practically eliminates this adjustment of volumes.

The curve in figure 2 can be expressed in the following equation.

$$y = \frac{s}{x} - \frac{(1-x)K}{x^2}$$

where y = height of "unknown" solution, x = ratio of solutions and s = height of standard solution.

By s is meant the reading of the nephelometer with the standard solution used as an "unknown," or, in other words, when the ratio of the solutions is unity. This serves to eliminate any errors due to faulty light, tubes, plungers, etc.

Where K = a constant, obtained by substitution of standardization values.

Therefore, if once the value of K is derived for *any given standard solution and height of the standard solution*, the nephelometric readings will give at once, upon calculation, the ratio of the solutions, and thus no further adjustment⁹ of volumes is necessary.

When the K obtained with one height of standard solution is compared with that of another height, it is found that K is proportional to the height of the standard solution, the equation then becomes for any heights of liquids within moderate limits.

$$y = \frac{s}{x} - \frac{s(1-x)k}{x^2}$$

$$\text{where } k = \frac{K}{s}$$

One has, therefore, the choice of using either one or more standard curves and getting the ratios directly from a curve (as in figure 2), without calculation, or taking any suitable standard, adjusting either or both heights of liquids until the amount of light is matched, and obtaining the ratios by substitution in the nephelometric formula given above. A more convenient form when expressed in terms of y , is

$$x = \frac{s + sk + \sqrt{(s + sk)^2 - 4s ky}}{2y}$$

⁹ Of course, it is assumed that solutions which differ from a standard solution very much as may easily be observable macroscopically, will require, as in colorimetric work, a more suitable standard.

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when s is kept constant at 14.96 as in the curve, figure 2, and when K or Sk has a value of 1.8 the equation becomes

$$x = \frac{16.76 + \sqrt{280.9 - 7.2y}}{2y}$$

Edestin ratios.

10 5 RATIO 1* y	12 5 RATIO 0.882 y	15 5 RATIO 0.750 y	20 5 RATIO 0.600 y	22.5 5 RATIO 0.545 y	25 5 RATIO 0.500 y	28 5 RATIO 0.429 y
14.9	16.7	19.2	23.1	24.9	26.3	28.8
14.9	16.8	19.3	23.0	24.6	25.9	29.5
15.1	16.5	19.2	22.9	25.0	26.0	28.7
15.1	16.6	19.1	23.4	24.83	26.0	28.5
14.9	16.64	19.1	23.0	average	26.0	28.7
15.0	average	18.9	22.6		26.0	28.6
15.1		19.0	22.8		26.1	28.5
15.0		18.9	22.8		26.04	29.0
15.0		19.0	23.1		average	28.8
15.0		19.0	22.7			29.0
15.0		19.1	23.2			28.81
15.0		19.2	23.2			average
15.0		18.9	23.0			
14.9		19.3	23.3			
14.9		19.3	23.0			
14.9		19.2	22.9			
15.0		19.2	23.00			
14.9		19.4	average			
15.0		19.13				
14.9		average				
14.8						
15.1						
14.9						
15.0						
14.9						
14.9						
15.1						
14.96						
average						

*s = 15.0

The table on p. 492 gives the values obtained with edestin when 5 cc. of a 0.01 per cent solution, diluted with 10 cc. of sodium chloride reagent, was used as a standard.

The numerator of the fraction indicates the number of cubic centimeters of the reagent which was used to dilute the protein solution: the denominator indicates the number of cubic centimeters of 0.01 per cent edestin solution used. The third row of figures indicates the calculated ratios. The figures in columns are interlined to indicate the number of readings per solution. The average of all the figures of a column is given at the foot of each column, and is also indicated by crosses in figure 2. As may be seen, down to the ratio 0.50 they follow the curve quite satisfactorily, but beyond this ratio they deviate slightly, owing to a factor that is produced by a difference in the height of the liquids. In actual work this may easily be eliminated by following the curve of observed readings, or by choosing a more comparable standard. It may also be taken into account in calculating the ratios, as illustrated in the following paragraphs.

This factor that causes the nephelometric readings to be slightly too low, especially when the ratios are below 0.500 and when the per cent of edestin of any of the solutions is below 0.0033 per cent, is due to the fact that even pure water will produce some light in the eyepiece. This arises, no doubt, from imperfections of the apparatus, it being very difficult to exclude all stray reflection of light. The equivalent of this amount of light is estimated by making a control determination on pure water, using a 0.0005 per cent solution of edestin as a standard. Under the conditions given in this paper, 28.0 mm. of pure water matched a 0.0005 per cent solution of edestin at 5.4, 4.7, 4.5, 4.8, and 5.7 mm. = 5.0 mm. average. Assuming this proportion to hold for all heights of distilled water, we find that 23 mm. of water will give the same amount of light as 5.0 mm. of 0.0005 per cent edestin (excluding the amount of light from its 5 mm. of water; thus $28.0 - 5.0 = 23.0$ mm.) and therefore 1.0 mm. of water = 0.21 mm. of 0.0005 per cent edestin. Hence, when very weak solutions are determined, care should be used to see that control corrections are applied, or eliminated by careful standardization.

In order to test the general applicability of the nephelometric formula given above, other heights of solutions and ratios than those covered in the curve were used as is shown in the table below:

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STANDARD OR CONCENTRATION OF "x"		"UNKNOWN" OR CONCENTRATION OF "y"		MM. OF "x"	MM. OF "y"	THEORETICAL RATIO $\frac{x}{y}$	FOUND IF $\frac{x}{y} = \frac{z}{y}$	$\frac{z}{y} = \frac{x}{y}$	$\frac{(x-y)^2}{(x+y)^2}$
(1-3) NaCl	0.01 per cent edestin	(1-3) NaCl	0.01 per cent edestin						
cc.	cc.	cc.	cc.						
10	5	25	5	15.1	26.9	0.500	0.562	0.489	
				10.0	18.2	0.500	0.549	0.477	
10	5	25	5	14.8	25.9	0.500	0.571	0.504	
				9.9	17.6	0.500	0.562	0.493	
				8.0	14.0	0.500	0.571	0.495	
10	5	25	5	15.1	26.8	0.500	0.563	0.494	
				12.0	21.8	0.500	0.550	0.488	
				17.0	29.9	0.500	0.568	0.500	
5	5	15	5	15.2	27.5	0.500	0.554	0.472	
				15.2	26.8	0.500	0.568	0.489	
Average.....						0.500	0.562	0.491	
				Observed y	Corrected y				
25	5	45	5	28.9	13.0	32.3	0.300	0.450	0.275
				11.1	5.0	12.3	0.300	0.451	0.284
25	5	95	5	22.1	10.0	24.6	0.300	0.450	0.282
				28.5	13.0	31.7	0.300	0.456	0.289
95	5	pure H ₂ O		5.2	28.0				
				4.8	28.0				
Average.....						0.300	0.452	0.282	

These results are sufficient to show that by the use of either the formula within moderate limits or curves prepared from three or four sets of readings, one can use the nephelometer for the determination of suspended protein and organic substances with the accuracy and with the ease with which the best colorimetric work is done.

Since this nephelometric formula seems to hold for a protein of high molecular weight, it was of interest to try its application to argentic chloride suspensions. In order to avoid the corrections necessary when very weak solutions are compared, as indicated above with edestin, relatively strong solutions were used.

STANDARD "x"		"UNKNOWN" "y"		MM. OF "x"	MM. OF "y"	THEO- RETICAL z	z FOUND IF $z = \frac{s}{y}$	$z = \frac{s}{y}$ z FOUND IF $z = \frac{s + ab + \sqrt{(s+ab)^2 - 4abx}}{2y}$ AND $b = 0.21$
H ₂ O	0.00872 per cent AgCl	H ₂ O	0.00872 per cent AgCl					
cc.	cc.	cc.	cc.					
	10		10	15.0	15.0 15.1 15.0 15.0	1.00	1.00	1.00
	10	10	10	15.0	23.8 23.8 23.6 23.5 23.7	0.500	0.633	0.500
10	10	30	10	15.0	23.9 23.9 23.9 23.9	0.500	0.628	0.491
	0.001432 per cent AgCl		0.001432 per cent AgCl					
	10		10	15.0	15.1 15.0 15.0 15.0	1.00	1.00	1.00
	10	10	10	15.0	23.6 23.4 23.5 23.5 23.5	0.500	0.638	0.509

As may be seen from the results with argentic chloride the nephelometric formula holds for considerable variations in concentrations; the strongest solution being eight times as strong as the weakest, or a ratio of 0.125. It is probable that when corrections are made, especially for the weaker solutions, the result will be still more consistent with the formula. It is to be noted that these experiments give $k_{\text{AgCl}} = 0.21$ and $k_{\text{EDESTIN}} = 0.12$.

(c) *Solutions and reagents.*

Edestin solutions were made as follows:

1. *Stock solution:* 0.1000 gram of Merck's edestin, weighed accurately in a 50 cc. beaker, was moistened with sufficient water to make it possible to rub the substance into a paste. After thorough rubbing, 3 cc. of $\frac{N}{10}$ hydrochloric acid was added which dissolved all but a trace of the suspended substance. After allowing the solution to settle for an hour, it was filtered through a well-washed filter paper into a clean, 100 cc. graduated flask. This was done to prevent foreign particles and fibers of filter-paper from getting into the solutions. On bringing the solution up to the mark, adding a few drops of chloroform, shaking and allowing it to stand for twenty-four hours, it was ready for use.

2. *Standard solution:* 10 cc. of this stock solution were diluted to 100 cc., thus making it a 0.01 per cent solution. One volume of this standard solution, with one, two or more volumes of 1-3 NaCl solution gave suitable standards for nephelometric work. These standards will remain as suspensions one or more hours depending on the dilution. In the work given above a fresh standard was made for every determination.

3. *Precipitant for acid edestin or edestan:* (1-3) NaCl solution.

a. *Stock solution:* A saturated solution of sodium chloride.

b. *Reagent. (1-3) NaCl:* One volume (250 cc.) of saturated NaCl solution, with three volumes of water (750 cc.) was found to give satisfactory results in precipitating the edestan completely.

(d) *Directions.*

For precipitating substances. Thus far, satisfactory results have been obtained by adding the precipitant drop by drop from a carefully standardized burette to the standard solutions contained

in a beaker or small Erlenmeyer flask. Test tubes should be avoided as it is difficult to shake the solution properly, without adding a stopper or introducing minute air-bubbles into the solution. An Erlenmeyer flask or beaker shaken in a rotatory fashion gently is most satisfactory.

The tubes may be cleaned and dried with alcohol and ether, or any other suitable way so long as no dust or lint is thereby introduced.

For reading the instrument: The plunger, being painted with asphaltum paint, obviously cannot be cleaned and dried with alcohol and ether; but may be rinsed with some of the liquid to be examined, or may be wiped carefully with a lint-free cloth or lens paper. To use the instrument, it is well to insure proper working conditions by putting into both tubes some standard liquid and comparing the heights. If the readings are consistent and practically equal on both sides, one may presume the lighting arrangements and other parts are in proper adjustment.

The following suggestions were helpful in obtaining good results.

1. The instrument was kept in a separate room, where all the lights could be extinguished at pleasure.

2. After the tubes containing the suspensions were in position, the room was darkened completely for a few minutes, to rest the eyes. Using the instrument directly after coming from a well-lighted room produced inferior results.

3. Both eyes were kept open in reading the instrument. This was easily accomplished with the shaded eyepiece, and produced less strain.

4. It was found advisable not to make the adjustments too rapidly or too constantly in order to avoid inaccuracies due to eye-strain. The final adjustment was made only after relaxing the eyes for a few minutes. The eyes were used alternately, the approximate adjustment was made with one eye and the final always with the other.

5. When much nephelometric work was done necessitating going to and from a strongly lighted room, often, smoked glasses were found restful and advantageous.

SUMMARY.

I. A micro-chemical method for following the digestion of a soluble protein, edestin, based on the use of a nephelometer is given.

II. A new and sensitive nephelometer, easily made from and into a Duboscq colorimeter is described.

III. The readings of the nephelometer plotted against the ratios of the solutions, for a given standard solution and a given height of standard, seem to follow a uniform curve which can be expressed in the equation $y = \frac{s}{x} - \frac{s(1-x)k}{x^2}$ where y = height of "unknown" solution, s = height of standard solution, x = ratio of solutions.

The studies of various precipitants for protein and other organic substances in dilute solutions are in progress with the view of extending the application of this method generally. The ease, the rapidity and the accuracy of the method would make it very useful, if the proper precipitants can be found. By proper dilution it can be used for large amounts of substances, and is sensitive enough to determine 0.00002 gram with a percentage error of less than 2 per cent. The determination of casein in milk and the estimation of minute quantities of ricin are receiving immediate attention.

THE PREPARATION AND PROPERTIES OF A COMPOUND PROTEIN; GLOBIN CASEINATE.

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I have elsewhere pointed out¹ that the investigations of Hardy² indicate that the proteins in blood sera are united to form a complex which, we may imagine, bears a somewhat similar relation to the individual proteins which constitute it as the proteins bear to the polypeptides out of which they are constructed, and I have furthermore suggested that the biochemical and immunological specificity which is displayed by animal fluids and tissues may possibly be attributable, directly or indirectly, to the existence of these complexes.

The only compounds of proteins with one another which have so far been isolated and studied in detail are those which the various protamines form with such proteins as egg albumin, gelatin, hemi-elastin, casein and edestin.³ These compounds are formed on bringing the two proteins together in weakly alkaline (ammoniacal) solution (Kossel). Gay and Robertson have pointed out, however, that it is necessary to have present an excess of the protamine component, otherwise the compound does not readily flocculate.⁴ Hunter describes clupeine caseinate, which may serve as a type of these compounds, as being insoluble in cold and very slightly soluble in hot water, somewhat soluble in dilute acids and readily precipi-

¹ T. Brailsford Robertson: *Univ. of Calif. Publ. Physiol.*, iv, p. 25, 1911; *Die physikalische Chemie der Proteine*, Dresden, 1912, p. 126.

² W. B. Hardy: *Journ. of Physiol.*, xxxiii, p. 251 (Appendix), 1905.

³ A. Kossel: *Deutsch. med. Wochenschr.*, vii, 1904, cited after A. Hunter: *Zeitschr. f. physiol. Chem.*, liii, p. 527, 1907.

⁴ F. P. Gay and T. Brailsford Robertson: *Journ. of Exp. Med.*, xvi, p. 479, 1912.

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tated from these solutions by ammonia. It is soluble in salt solution. On digestion with pepsin-HCl, the casein moiety undergoes hydrolysis, depositing paranuclein, while the protamine moiety remains unhydrolyzed. In all of the compounds of clupeine with various proteins which Hunter investigated, the combining weight of the protamine was always considerably smaller than that of the other component of the compound.⁷ Thus 2.5 parts of casein combine with 1 part of clupeine to form clupeine caseinate, while the compound of clupeine with edestin contains no less than 8.5 parts of edestin by weight to every one of clupeine.

Globin is generally considered to be a member of the histone group of proteins,⁸ and, according to Kossel,⁶ the histones are built up around a large protamine nucleus to which they owe their pronounced basic qualities. One might anticipate, therefore, that globin would behave analogously to the protamines and form similar compounds with the less basic proteins.

I find that if casein dissolved in dilute alkali be mixed with excess of globin, similarly dissolved in dilute alkali, a coagulum-like precipitate results which would appear, by its behavior, to be a compound of globin with casein, analogous in many respects to the protamine caseinates. The method employed in preparing this substance in bulk was as follows:

The globin was prepared from ox-blood corpuscles by a method which I have described in detail elsewhere in this number.⁷ The preparation employed in these experiments (designated Preparation II in the communication cited) is somewhat tinged with haematin and yields somewhat deeply colored, brown solutions (in 2 per cent concentration). The casein was Eimer & Amend's C. P. Casein "nach Hammarsten," specially purified by a method which has been described elsewhere.⁸

In preliminary experiments it was found that, in order to bring about the formation of a precipitate in a globin-casein mixture, it was necessary to avoid the presence of an excess either of casein

⁷ Fr. N. Schulz: *Zeitschr. f. physiol. Chem.*, xxiv, p. 449, 1898.

⁸ A. Kossel and F. Kutscher: *Zeitschr. f. physiol. Chem.*, xxxi, p. 165, 1900; A. Kossel and H. Pringle: *ibid.*, xlix, p. 301, 1906; A. Hunter: *loc. cit.*

⁷ T. Brailsford Robertson: this *Journal*, xiii, p. 455, 1913.

⁸ T. Brailsford Robertson: *Die physikalische Chemie der Proteine*, Dresden, 1912, p. 32.

or of alkali. Rough estimations led to the impression that, given a favorable alkalinity,⁹ the proportion of globin to casein should be about 2 to 1 in order to secure a maximum yield of the precipitate.

Globin (1.5 grams per 100 cc. of solvent) dissolves extremely slowly in $\frac{N}{50}$ or $\frac{N}{30}$ KOH solution, while it readily and completely dissolves in $\frac{N}{10}$ KOH and the concentration of KOH may then be reduced to $\frac{N}{50}$ by the addition of HCl, while stirring, without causing any permanent precipitate to appear.¹⁰ On the other hand, if two and a half volumes of a 1.5 per cent solution of globin in $\frac{N}{30}$ KOH be added to one volume of a 2 per cent solution of casein in 0.016 N KOH (neutral to phenolphthalein), no precipitate is obtained because the excess of alkali dissolves the globin caseinate, while, if two and a half volumes of a 1.5 per cent solution of globin in $\frac{N}{50}$ KOH be added to a similar solution of casein, a copious precipitate results. Accordingly, in preparing the compound it is advisable to dissolve the globin in a slight excess of alkali, and neutralize a portion of this alkali before mixing the solution with the solution of casein.

Three hundred and fifty cc. of $\frac{N}{10}$ KOH were diluted to 1200 cc. and 20 grams of globin were dissolved therein. When solution was complete (one to two hours) 150 cc. of $\frac{N}{10}$ HCl were added to the mixture while stirring. A clear brown solution was thus obtained.

Ten grams of casein were dissolved in 500 cc. of 0.016 N KOH. These two solutions were then mixed, the mixture diluted to 7 liters by the addition of distilled water, thoroughly shaken, and then allowed to settle in tall glass cylinders over night. The supernatant fluid was then decanted from the heavy brownish coagulum-like precipitate, which became more granular in the subsequent washings. The decanted fluid was very opaque and obviously contained much finely suspended protein material.

The precipitate was washed by decantation in 40 liters of distilled water in six successive washings. The final washings were perfectly clear. It was then washed in 10 liters of absolute alcohol

⁹ A slight excess of alkali readily redissolves the precipitate.

¹⁰ Analogous phenomena are displayed by casein and other proteins. Cf. T. Brailsford Robertson: *Die physikalische Chemie der Proteine*, Dresden, 1912, p. 70.

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in two successive washings, collected upon a hardened filter, washed with 2 liters of ether within an incubator over H_2SO_4 and dried in the incubator at 37°C . for twenty-four hours. It was then pulverized and sifted and returned to the incubator and dried over H_2SO_4 at 37°C . for three days. The substance was thus obtained in the form of a coarse brownish-gray powder, the yield being 7.5 grams.

The phosphorus in two 1-gram portions of this material was determined by von Wendt's modification of Neumann's method¹¹ with the following results:

NUMBER OF SAMPLE	MGM. P_2O_5 IN 1 GRAM	PER CENT OF PHOSPHORUS
Sample 1.....	7.16	0.313
Sample 2.....	6.86	0.300
Average.....		0.306

The percentage of phosphorus in casein is 0.887,¹² hence the proportion of casein contained in this preparation of globin caseinate is 34.5 per cent.

This preparation of globin caseinate is readily soluble in dilute alkalis, yielding brownish solutions, and in dilute acids, yielding light-colored solutions. It is not soluble in distilled water, and the globin caseinate is precipitated from alkaline solutions by neutralization with acid. Excess of acid, even of acetic acid, redissolves this precipitate, thus indicating very clearly that the casein is not present as such in a condition of mechanical admixture. Globin similarly is precipitated from alkaline solutions by neutralization, but that the properties of the globin have been very materially modified by combination with casein is shown by the *excess* of acetic acid which is necessary in order to redissolve the precipitate which occurs upon neutralization. Thus, to 43 cc. of an approximately 1.25 per cent solution of globin caseinate in $\frac{\text{N}}{16}$ KOH, $\frac{\text{M}}{6}$ acetic acid solution was added slowly. After adding 19 cc. the substance still remained in solution, then a precipitate began to appear, which still persisted until over 65 cc. of the acid solution

¹¹ G. von Wendt: *Skand. Arch. f. Physiol.*, xvii, p. 217, 1905.

¹² O. Hammarsten: *A Text-book of Physiological Chemistry*, trans. by J. A. Mandel, 6th Ed., New York, 1911, p. 615.

had been added, when a very opaque light-colored solution was obtained. To 43 cc. of a 1.25 per cent solution of globin (Preparation II, cf. above) $\frac{M}{6}$ acetic acid was similarly added, a permanent precipitate had already appeared after the addition of 16 cc. of the acid solution, and 25 cc. sufficed to completely redissolve it. I continued adding $\frac{M}{6}$ acetic acid until 65 cc. had been added. A clear brown solution was obtained.

It is clear, therefore, that by the union with casein the basic qualities of globin have been decreased and its acid function enhanced. Globin caseinate is held in solution by a smaller excess of alkali than that required to hold the same amount of globin in solution, while a greater excess of acid is required to carry globin caseinate into solution than to carry globin into solution. On the other hand, the acid qualities of casein have been diminished and its basic function enhanced by the union with globin, for no excess of dilute ($\frac{M}{4}$ or more dilute) acetic acid solution will carry any casein into solution; a stronger acid, such as a mineral acid, is required. It is evident that in these respects the properties of globin caseinate are intermediate between those of globin and those of casein.

The solution of globin caseinate in dilute acetic acid does not deposit any precipitate even on standing at 55°C. for twenty-four hours. It does, however, yield a precipitate on boiling for two or three minutes, and this precipitate does not redissolve on cooling; it is not a coagulum of heat-denatured protein, for it is soluble in dilute alkali. It is probably, therefore, casein, and we may conclude that globin caseinate is decomposed into its components by boiling in dilute acid solution.

To 70 cc. of each of the above solutions of globin caseinate and of globin, respectively, in dilute acetic acid were added 5 cc. of a 1 per cent solution of Grüber's pepsin puriss. sicc., and the mixtures were placed in an incubator at 37°C. After two hours a very slight flocculent precipitate had appeared in the globin solution and a dense granular precipitate in the globin caseinate solution which left the supernatant fluid clear. After twenty-four hours, the dense precipitate in the globin caseinate digest had completely redissolved and in each mixture there remained only the slight flocculent precipitate first noted in the globin digest. The dense precipitate in the globin caseinate digest cannot have been para-

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nuclein resulting from partial hydrolysis of casein, for paranuclein, once precipitated in peptic digests, does not redissolve as digestion proceeds.¹³ Hence we may conclude that in all probability it was casein, and that the first stage in the action of pepsin upon globin caseinate is to decompose it into its components—globin and casein—which subsequently undergo hydrolysis separately.

The globin caseinate digest, after twenty-four hours' digestion, contained no casein either combined or uncombined with globin, as it yielded no precipitate on boiling.

The following table of comparative precipitation reactions displays certain respects in which the behavior of globin caseinate resembles or differs from that of casein or of globin. The solutions employed were all 2 per cent solutions in $\frac{N}{10}$ KOH. A + indicates precipitation (or coagulation) and a 0 indicates no precipitation or coagulation:

REAGENT	GLOBIN	GLOBIN CASEINATE	CASEIN
Dilute acetic acid (cold).....	0	0	+
Dilute acetic acid (boiling).....	0	+	+
Half saturated Am_2SO_4	+	+	+
Four volumes of absolute alcohol....	0	0	0
Trichloroacetic acid.....	+	+	+
5 per cent NaCl.....	0	0	0
Half saturated Na_2SO_4	0	0	0
Saturated NaCl.....	+	+	0

(+ on heating in the presence of excess of the salt)

I prepared 2 per cent and 1 per cent solutions of globin caseinate in $\frac{N}{10}$ KOH and determined the refractivities of these solutions and of $\frac{N}{10}$ KOH at 16° in a Pulfrich refractometer, reading to within 1' of the angle of total reflection. A sodium flame was the source of light. The following were the results obtained. The values bracketed are calculated from the formula $n - n_1 = a \times c$, where

¹³ Lubavin: Hoppe-Seyler's *Med.-Chem. Untersuch.*, Berlin, 1866, p. 463; A. Kossel: *Verhandl. d. Berl. physiol. Gesellsch.*; *Arch. f. (Anat. u.) Physiol.*, 1901, p. 181; T. Brailsford Robertson: this *Journal*, iii, p. 95, 1907.

n is the refractive index of the solution, n_1 that of the solvent and c is the percentage of protein in the solution.

SOLUTION	n = REFRACTIVE INDEX OF SOLUTION AT 16° C.	a
$\frac{1}{15}$ KOH	1.33552	
1 per cent globin caseinate.....	1.33711	0.00159 ± 0.00008
2 per cent globin caseinate.....	1.33879	0.00164 ± 0.00004

A duplicate set of determinations yielded identical results.

In order to assign to each observation its due weight in determining the average value of a for each of these preparations, it is necessary to add together the observed values of $n - n_1$ for the two solutions and divide this sum by the sum of the concentrations employed ($= 3$). Proceeding in this way ¹⁴ we obtain:

$$a = 0.00162 \pm 0.00005.$$

Now the value of a ($=$ change in refractive index of solvent due to 1 per cent of protein) for casein is 0.00152,¹⁵ while the value of a for globin is 0.00169.¹⁶ Hence if the refractivity per gram per 100 cc. of solution of globin caseinate were the algebraic sum of the separate refractivities of its components, since, as we have seen, globin caseinate contains 34.5 per cent of casein, the value of a for globin caseinate should be:

$$\frac{34.5 \times 0.00152 + 65.5 \times 0.00169}{100} = 0.00163$$

which is identical, within the experimental error, with the value experimentally ascertained.

I have elsewhere shown¹⁷ that the refractivity of the mixed (or combined) proteins of blood sera is equal to the sum of the refractivities of the separate constituent proteins, and, as I have stated, there is much reason to believe that these proteins are bound together to form a compound protein complex. We may

¹⁴ T. Brailsford Robertson: *Die physikalische Chemie der Proteine*, Dresden, 1912, Chapter 13.

¹⁵ T. Brailsford Robertson: *Journ. of Physical Chem.*, xiii, p. 469, 1909.

¹⁶ T. Brailsford Robertson: *this Journal*, xiii, p. 462, 1913.

¹⁷ T. Brailsford Robertson: *this Journal*, ix, p. 179, 1912.

506 Preparation and Properties of Globin Caseinate

therefore conclude that *the refractivity of a compound protein is an additive function of the refractivities of its components.*

CONCLUSIONS.

1. Globin caseinate may be prepared by mixing two parts by weight of globin with one part by weight of casein, each in faintly alkaline solution.

2. Globin caseinate in aqueous solution displays properties intermediate between those of globin and casein, indicating that the acid function of globin is enhanced by union with casein and the basic function of casein by union with globin.

3. Globin caseinate is not decomposed by dilute acetic acid in the cold. It is decomposed into globin caseinate by the action of boiling dilute acetic acid or by a brief action of pepsin in the presence of dilute acetic acid.

4. The change in the refractive index of $\frac{N}{10}$ KOH due to the introduction of 1 per cent of globin caseinate is 0.00162 ± 0.00005 .

5. The refractivity of a compound protein is an additive function of the refractivities of its components.

ON NUCLEASES.

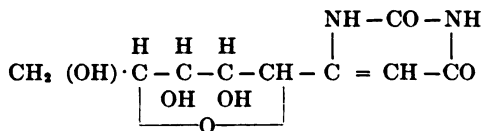
THIRD PAPER.

By P. A. LEVENE AND F. B. LA FORGE.

(From the Laboratories of the Rockefeller Institute for Medical Research,
New York.)

(Received for publication, December 4, 1912.)

It was demonstrated through the work of Levene and Jacobs¹ that, compared with purine ribosides, the pyrimidine ribosides possessed a much higher resistance towards the hydrolytic action of mineral acids, and on the basis of this property a method was devised for the isolation of pyrimidine bodies. It was later shown by Levene and Medigreceanu² that a similar difference existed between the two classes of ribosides in regard to their behavior towards certain enzymes. Thus it was found that nucleosidases, which hydrolyzed the purine derivatives, remained without action on the pyrimidine bodies. In a previous work by³ the present writers the fact was brought to light that the difference in the behavior of the two classes of substances towards dilute mineral acids can be removed by the reduction of the pyrimidine base in the pentoside to the corresponding dihydropyrimidine. Thus, while uridine was resistant to boiling with dilute aqueous mineral acids, dihydro-uridine behaved under these conditions similarly to inosine, or adenosine, or to any other glucoside.

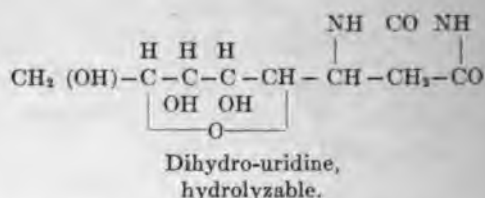


Uridine,
non-hydrolyzable by dilute acids.

¹ Levene and Jacobs: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3150, 1910.

² Levene and Medigreceanu: *this Journal*, ix, p. 65, 1911; ix, p. 389, 1911.

³ Levene and La Forge: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 608, 1912.



Thus, dihydro-uridine was shown to possess a chemical structure in every way analogous to other nucleosides, and hence presented a convenient medium for the study of the specificity of nucleosidases. Thus, if it were possible to find tissue extracts which would hydrolyze the nucleosides of one group and failed to decompose the substances of the other group, this find would contain the proof of the existence of specific enzymes capable of acting on nucleosides of only one definite group. In reality it was found that the tissue that possessed the most pronounced action on the purine nucleosides remained without action on dihydro-uridine, and as yet it is impossible to find an enzyme capable of hydrolyzing either the original uridine, or dihydro-uridine. Hence, the mechanism by which the organism brings about the dissolution of pyrimidine nucleosides is as yet undiscovered, but it is made certain that individual nucleosidases possess a limited specific activity.

EXPERIMENTAL PART.

In the experiments here recorded the extract of the intestinal mucosa was used as an enzyme solution. It was prepared in the manner described in the papers by Levene and Medigreceanu.

The activity of the extract was tested on adenosine solution and on dihydro-uridine. The presence or absence of hydrolysis was ascertained by the changes in the optical activity of the solution in different intervals after the beginning of the experiment and by the power of the reaction product to reduce Fehling's solution.

The solutions of enzyme and nucleoside were made up to contain one per cent of Henderson's phosphate mixture.

Toluene was used as antiseptic in all experiments.

Experiment 1. (a) About 0.4 gram of dihydro-uridine was dissolved in 7.0 cc. of water, 1.0 cc. of a 10 per cent Henderson's phosphate mixture and 2.0 cc. of the enzyme solution were added.

Original rotation, $[\alpha]_D = -0.50^\circ$

After 24 hours, $[\alpha]_D = -0.50^\circ$

(b) 4.0 cc. of a cold saturated aqueous solution of adenosine were diluted with 0.5 cc. of a 10 per cent phosphate mixture and 0.5 cc. of the enzyme solution.

Initial rotation, $[\alpha]_D = -0.45^\circ$

After 24 hours, $[\alpha]_D = -0.20^\circ$

(c) 1.0 cc. of Henderson's phosphate solution + 2 cc. of enzyme solution and 7 cc. of water.

Initial rotation, $[\alpha]_D = +0.05^\circ$

After 24 hours, $[\alpha]_D = +0.05^\circ$

The dihydro-uridine solution gave very slight reduction on prolonged boiling with Fehling's solution. An increase in the reducing power at the end of the experiment could not be noticed. The adenosine solution showed no reduction at the beginning of the experiment and a marked reduction at the end.

The enzyme solution had no reducing power.

Experiment 2. (a) To 5 cc. of a 25 per cent solution of dihydro-uridine was added 0.5 cc. of the enzyme solution.

Initial rotation, $\alpha_D = -3.82 (\pm 0.02)$

After 48 hours, $\alpha_D = -3.88 (\pm 0.02)$

(b) Enzyme solution did not possess any appreciable optical activity.

(c) About 1.0 gram of adenosine was suspended in 20.0 cc. of 1 per cent phosphate mixture and 2.0 cc. of enzyme solution; since a part remained insoluble it was not possible to measure the optical rotation of the substance. The action of the enzyme solution on adenosine was ascertained by the reducing power of the solution on Fehling's solution.

The dihydro-uridine showed no change in reducing power before and after enzyme action.

Adenosine solution possessed no reducing power on Fehling's solution at the beginning of the experiment and a very marked reduction after forty-eight hours.

The enzyme solution showed no reducing power either at the beginning or at the end of the experiment.



THE BIOCHEMISTRY OF THE FEMALE GENITALIA.

II. THE LIPINS OF THE OVARY AND CORPUS LUTEUM OF THE PREGNANT AND NON-PREGNANT COW.¹

By JACOB ROSENBLOOM.

(From the Laboratory of Biochemistry of the University of Pittsburgh, Pittsburgh, Pa.²)

(Received for publication, December 4, 1912.)

Introduction. The biochemistry of the female genitalia is a subject of which practically nothing is known. This paper contains the data of a study of the lipins of the ovary and of the corpus luteum of the cow in pregnant and non-pregnant conditions.

Methods. The desiccation of the material, extraction of the lipins and the chemical analyses were carried out according to the methods described in a former paper³ with the addition that the cholesterol and cholesterol esters were determined by the excellent method of Windaus.

The table on the following page contains the results obtained in this investigation.

Conclusions. 1. Data are presented showing the percentages of lipins, phospho-lipins, neutral fat, fatty acids and cholesterol in the ovary and corpus luteum of the cow in the non-pregnant state and in the pregnant state.

2. The results indicate that there is practically no increase in the above mentioned substances during pregnancy in the cow.

¹ A preliminary account of the investigation was published in *Science*, xxxiv, p. 222, 1911; *Biochem. Bull.*, i, p. 115, 1911.

² Most of the analytic data presented in this paper were obtained while working in the Laboratory of Biological Chemistry of Columbia University at the College of Physicians and Surgeons.

³ Hanes and Rosenbloom: *Journ. of Exp. Med.*, xiii, p. 355, 1911.

Table showing lipin composition of ovary and corpus luteum of the pregnant and non-pregnant cow.

DRY WEIGHT OF TISSUE	LIPINS		PHOSPHOLIPINS		NEUTRAL FAT		FATTY ACIDS		CHOLESTEROL		CHOLESTEROL ESTERS
	grams	per cent	In lipin extract	In dry tissue	In lipin extract	In dry tissue	In lipin extract	In dry tissue	In lipin extract	In dry tissue	
			per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
<i>A. Non-pregnant ovary, without the corpus luteum.</i>											
20.7555	1.426	6.87	57.41	3.94	9.99	0.755	25.9	1.72	6.70	0.446	trace
<i>B. Pregnant ovary, without the corpus luteum.</i>											
(1)* 5275	1.838	6.23	55.88	3.48	9.67	0.60	28.10	1.75	6.35	0.40	trace
(2)† 6400	1.320	6.39	56.87	3.63	9.87	0.63	26.42	1.69	6.84	0.437	trace
<i>C. Non-pregnant corpus luteum.</i>											
11.201	3.029	27.04	52.14	14.10	10.90	2.95	32.94	8.91	4.02	1.09	trace
<i>D. Pregnant corpus luteum.</i>											
(1)* 10.250	2.820	27.51	52.31	14.90	11.11	3.05	32.62	8.97	3.96	1.09	trace
(2)† 8.364	2.3405	27.95	53.20	14.87	10.69	2.99	32.01	8.95	4.20	1.17	trace
<i>E. Ovary with corpus luteum (non-pregnant)—Averages of A and C.</i>											
			16.95	54.77	9.02	10.44	1.85	29.42	5.31	5.36	0.77
<i>F. Ovary with corpus luteum (pregnant)—Averages of B and D.</i>											
(1)*	16.87	54.09	8.94	10.39	1.82	30.36	5.30	5.15	0.74	0.80	trace
(2)†	17.17	55.03	9.25	10.28	1.81	29.22	5.32	5.52	0.80	0.80	trace

* Length of foetuses, 10-60 cms.

† Length of foetuses, 80-130 cms.

THE FATE OF PROLINE IN THE ANIMAL BODY.

By H. D. DAKIN.

(From the Herter Laboratory, New York.)

(Received for publication, December 9, 1912.)

Since nothing appeared to be known as to the fate of proline in the animal body, it seemed desirable to make some experiments in the hope that some light might be thrown upon the changes which this amino-acid may undergo.

As a first step in the investigation, observations were made on the behavior of proline when added to blood used for perfusing a surviving dog's liver, using essentially the method which has been so successfully applied by Embden, and secondly, an examination was made of the influence of proline on the glycosuria and acidosis of dogs under the influence of phlorhizin.

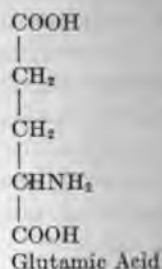
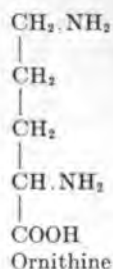
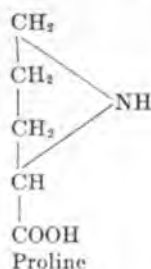
Proline, when added to blood used for perfusing a surviving dog's liver, leads to no increase in the normal formation of acetoacetic acid, nor is the acetoacetic acid excretion of glycosuric animals markedly increased by the administration of proline. It is clear, therefore, that proline is not to be classed with phenylalanine, tyrosine or leucine in having acetoacetic acid for a common catabolic path.

On the other hand, administrations of proline to the glycosuric animal was found to result in a marked increase in sugar output, so that it would appear that proline should be grouped with glutamic and aspartic acids, alanine and glycine, all of which have been shown by Lusk,¹ Ringer and others to be capable of furnishing glucose.

The formation of glucose from proline must necessarily involve disruption of the ring and it is of interest to recall the similarity in structure of the amino-acids, proline, glutamic acid and orni-

¹ *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

thine, each of which has five carbon atoms. Glutamic acid has been shown by Lusk to be capable of sugar formation in the glycosuric animal and preliminary experiments by the writer indicate that arginine and hence ornithine also possesses this property. It would appear, therefore, that these three amino-acids are closely related both structurally and in the changes they may undergo in the animal body.



Liver perfusion experiments.

The proline used in the following experiments was obtained from gelatin in the usual way. It was purified by repeated solution in alcohol, conversion into the crystalline copper salt and decomposition of the latter with sulphuretted hydrogen, followed by careful recrystallization. About two-thirds of the product was the racemic variety and one-third optically active.

The experiments on the effect of adding proline to blood used for perfusion of a surviving liver were made in the same way as in the case of experiments previously reported.²

ADDED SUBSTANCE	WEIGHT OF DOG	VOLUME OF PERFUSION FLUID	TIME OF PERFUSION	ACETOACETIC ACID FORMED
	<i>kgm.</i>	<i>cc.</i>	<i>minutes</i>	<i>mgm.</i>
2 grams proline.....	9	950	45	49
2 grams proline.....	11.5	1000	50	44
	11	1000	50	51*

* Average of several experiments.

² This *Journal*, ix, p. 139, 1911.

Experiments with diabetic animals.

The customary experimental conditions carefully worked out by Lusk, in which a starving dog, rendered as free from glycogen as possible, is made fully diabetic by the administration every eight hours of phlorhizin dissolved in sodium carbonate, were not chosen for the following experiments. The reason for this is that a markedly increased acetoacetic acid excretion is apt to follow immediately the administration of the alkaline phlorhizin solution. This acetoacetic acid excretion may almost completely disappear after a couple of hours and then reappear on a subsequent injection of the alkaline solution. This tends to make the acetoacetic acid output irregular.

More suitable experimental conditions seemed to be offered by the method of phlorhizin administration described by Coolen³ to which my attention was drawn by Professor Lusk. It is found that if a single gram of finely powdered phlorhizin suspended in 7-8 cc. of olive oil be injected aseptically under the skin of a dog, a glycosuria of remarkable intensity and duration is produced. In the starving animal a Dextrose: Nitrogen ratio of approximately 3 is quickly established and remains substantially constant for several days.

A convenient method of experimenting appears to be as follows: A dog is given a gram of phlorhizin in olive oil and starved for forty-eight hours. A second gram of phlorhizin is then given and a trial determination of the D : N ratio is subsequently made. A third gram of phlorhizin is then given on the following day and the urine for the "fore-period" is then collected by catheter, washing the bladder out with warm 2 per cent boric acid solution. It is essential that the D : N ratio of this urine should not vary greatly from the preliminary trial, as it is useless to proceed with the administration of the amino-acid until a constant ratio has been established. The proline in each case was administered by subcutaneous injection of a concentrated aqueous solution.

The acetoacetic acid and acetone estimations were made by the customary iodine method. Glucose was determined by the gravimetric Fehling method and the results confirmed by polarimetric observation.

³ *Arch. de pharmacodynamie*, i, p. 267, 1895.

Experiment I. Weight of dog, 10 kgs.

PERIOD	HOURS	TOTAL NITROGEN	GLUCOSE	D N RATIO	ACETO- ACETIC ACID INCLUDING ACETONE	SUBSTANCE GIVEN
II	16	6.5	21.20	3.04 3.14	0.072	1 gram phlorhizin. 1 gram phlorhizin.
III.	12	6.94	26.13	3.76	0.042	{ 10.2 gms. proline = 1.24 gm. N.
IV	12	6.44	20.13	3.13	0.103	1 gm. phlorhizin.

Experiment II. Weight of dog, 6 kgs.

I				2.80		1 gram phlorhizin.
II	12	2.73	7.28	2.67	0.302	1 gram phlorhizin.
III	16	4.11	14.62	3.55	0.348	{ 10.03 gm. proline = 1.22 gm. N.
IV	12	2.47	6.88	2.79	0.475	1 gm. phlorhizin.
V				2.59		

In the first experiment, adopting a D : N ratio of 3.1, it is found that using Lusk's method of calculation, an extra glucose excretion of about 8 grams was observed to follow the administration of 10.2 grams of proline. In the second experiment, adopting D : N = 2.7, 10.03 grams proline gave "extra glucose" corresponding to about 7 grams. Lusk⁴ found an excretion of 13 grams of glucose to follow the administration of 20 grams of glutaminic acid, so that it would appear that a molecule of glutamic acid or proline may furnish approximately the same amount of glucose.

⁴ *Amer. Journ. of Physiol.*, xxii, p. 176, 1908.

SEASONAL VARIATION IN THE IODINE CONTENT OF THE THYROID GLAND.¹

BY ATHERTON SEIDELL AND FREDERIC FENGER.

(From the Hygienic Laboratory, U. S. Public Health Service, Washington, D. C., and the Research Laboratory in Organotherapeutics, Armour and Company, Chicago, Ill.)

(Received for publication, December 10, 1912.)

The numerous results which have so far been published upon the iodine content of the thyroid gland indicate a very considerable individual variation among animals even of the same species. It would therefore be expected that a seasonal variation in the iodine content could not readily be detected by analyzing composite samples of glands from a limited number of animals collected at various seasons of the year. In fact experiments along this line made by one of us² using thyroids from dogs and from sheep confirmed this conclusion. More recent determinations given in a paper by N. H. Martin³ have shown that composite samples of thyroids collected throughout the year from sheep slaughtered at Newcastle-on-Tyne, England, varied comparatively little in their content of iodine. A striking feature of the results however was the uniformly small size of the glands and the relatively high percentage of iodine.

¹ The results described in this paper are the outcome of an effort which was inaugurated by Dr. Reid Hunt of the Hygienic Laboratory to fix a standard for thyroid used in medicine. This need has been recognized by the Committee of Revision of the Pharmacopoeia and so far a tentative description of this product and a proposed iodine content of 0.2 per cent has been adopted. In gathering data for this proposed pharmacopoeial description several manufacturers generously supplied samples and information regarding the preparation of their products. Additional points arose and it appeared desirable to obtain further data upon the thyroid, particularly of animals other than sheep. Towards this end, therefore, coöperation between Armour and Company and the Hygienic Laboratory was secured and the results presented in the following pages were obtained.

² Seidell: this *Journal*, x, p. 95, 1911.

³ *Brit. and Colonial Druggist*, lxii, p. 99, 1912.

The experiments to be described in this paper were undertaken particularly to ascertain the extent of the variation in the iodine content of composite samples of the thyroids of animals used as food by man, rather than with the expectation of finding a seasonal regularity in this variation. The need of data of this character is evident since in the production of a thyroid preparation of constant physiological activity a knowledge of the variation in the iodine content of the thyroid glands which constitute the raw material for the manufacture of the drug is essential. Among other things, the results here shown demonstrate that a continued restriction of the source of pharmacopoeial desiccated thyroid to the glands of the sheep would make practically impossible the production in the United States of a drug of 0.2 per cent iodine content for all but a short period of the year.

The samples which furnish the material for the present paper were prepared from thyroid glands obtained in all cases from animals shipped to the Union Stock Yards in Chicago from practically all parts of the United States except the eastern and south-eastern States. In this way it can be inferred that local influences have been eliminated to a very large extent and the samples represent the average yield of nearly the whole country. The collection of the raw material and the preparation of the samples from it was carried out under the personal supervision of one of the writers in the following manner: About 2.5 pounds of healthy normal sized glands from adult sheep, beef and hogs were collected on Tuesdays and Thursdays of each week and stored at freezing temperature. At the end of two weeks the combined approximately 10-pound sample of glands from each of the three species of animals was freed from connective and other tissue by carefully trimming the individual glands by hand. The material was then finely minced and dried to constant weight at 50°C. on agate-ware trays. Grease was then removed by extraction with petroleum ether in a Soxhlet apparatus and the sample ground to a fine powder. The first eight samples of each series were ground by hand in a Wedgewood mortar to pass a 40-mesh sieve. All the other samples were ground in a small ball mill to pass a 100-mesh sieve. Of the thoroughly mixed powder composing each sample two portions of about 25 grams each were withdrawn; one to be retained in Chicago for analysis and one sent to the

Hygienic Laboratory in Washington. The determinations of the iodine were made by the one of us in Chicago from time to time as the samples were collected and independently by the other in Washington after all of the samples had been received. The Hunter⁴ method was used for both sets of determinations and the results which were obtained in each case were not communicated to the other party until both sets of determinations had been

TABLE I.

Sheep thyroids. Showing the percentage of iodine and moisture in dried thyroid glands of the sheep collected at bi-weekly periods during one year.

SAMPLE NO. AND MID-DATE OF ITS BI-WEEKLY COL- LECTION PERIOD	PER CENT OF IODINE AS DETERMINED BY:		PER CENT MOISTURE LOST AT 97.5°	PER CENT IODINE CALCU- LATED TO DRY BASIS
	Seidell	Fenger		
1911				
1 September 1.....	0.133	0.14	3.37	0.138
2 September 16.....	0.148	0.16	4.29	0.155
3 October 1.....	0.178	0.22	2.50	0.183
4 October 14.....	0.181	0.19	2.20	0.185
5 October 28.....	0.211	0.24	3.53	0.219
6 November 11.....	0.235	0.26	8.34	0.257
7 November 25.....	0.219	0.25	7.85	0.238
8 December 10.....	0.176	0.19	6.98	0.189
9 December 24.....	0.196	0.20	6.70	0.210
1912				
10 January 7.....	0.166	0.17	8.33	0.181
11 January 21.....	0.112	0.12	9.13	0.123
12 February 4.....	0.123	0.12	8.53	0.134
13 February 18.....	0.160	0.14	1.67	0.163
14 March 3.....	0.044	0.04	7.84	0.048
15 March 17.....	0.104	0.11	3.30	0.108
16 March 31.....	0.092	0.10	0.87	0.093
17 April 14.....	0.062	0.06	8.78	0.068
18 April 28.....	0.083	0.08	1.05	0.089
19 May 12.....	0.065	0.07	9.23	0.072
20 May 26.....	0.038	0.04	8.86	0.042
21 June 9.....	0.130	0.13	9.58	0.144
22 June 23.....	0.249	0.24	10.54	0.278
23 July 7.....	0.294	0.30	12.34	0.335
24 July 21.....	0.188	0.19	14.75	0.221
25 August 4.....	0.185	0.19	11.27	0.209
26 August 18.....	0.235	0.23	10.43	0.262

⁴ A. Hunter: this *Journal*, vii, pp. 321-349, 1910.

TABLE II.

Beef thyroids. Showing the percentage of iodine and moisture in dried thyroid glands of the beef collected at bi-weekly periods during one year.

SAMPLE NO. AND MID-DATE OF ITS BI-WEEKLY COL- LECTION PERIOD	PER CENT OF IODINE AS DETERMINED BY:		PER CENT MOISTURE LOST AT 97.5°	PER CENT IODINE CALCU- LATED TO DRY BARK
	Seidell	Fenger		
1911				
1 September 1.....	0.277	0.30	3.16	0.285
2 September 16.....	0.307	0.31	2.05	0.314
3 October 1.....	0.240	0.25	0.66	0.242
4 October 14.....	0.327	0.36	2.34	0.335
5 October 28.....	0.303	0.35	1.40	0.308
6 November 11.....	0.332	0.35	8.15	0.361
7 November 25.....	0.225	0.25	7.24	0.243
8 December 10.....	0.200	0.22	7.80	0.217
9 December 24.....	0.123	0.14	6.15	0.131
1912				
10 January 7.....	0.101	0.11	7.69	0.109
11 January 21.....	0.045	0.04	9.44	0.050
12 February 4.....	0.026	0.03	8.41	0.028
13 February 18.....	0.107	0.10	0.80	0.108
14 March 3.....	0.053	0.05	7.63	0.058
15 March 17.....	0.038	0.04	3.88	0.040
16 March 31.....	0.059	0.06	1.38	0.060
17 April 14.....	0.068	0.08	9.71	0.075
18 April 28.....	0.069	0.07	3.14	0.071
19 May 12.....	0.072	0.08	9.33	0.079
20 May 26.....	0.095	0.10	10.42	0.106
21 June 9.....	0.130	0.14	10.58	0.145
22 June 23.....	0.170	0.19	11.17	0.192
23 July 7.....	0.288	0.29	13.38	0.332
24 July 21.....	0.298	0.30	11.12	0.335
25 August 4.....	0.267	0.27	13.48	0.308
26 August 18.....	0.310	0.31	10.61	0.347

completed. The duplicate series of results are given in tables I, II and III and they furnish very gratifying evidence of the reliability of the Hunter method when applied independently by two analysts to quite a large number of samples. It will be noted that variations of more than 0.02 per cent iodine occur only among the first eight samples of each series. An explanation of these cases appears to be that these samples as mentioned above were coarsely ground and all the others were very finely ground and certainly most thoroughly mixed.

TABLE III.

Hog thyroids. Showing the percentage of iodine and moisture in dried thyroid glands of the hog collected at bi-weekly periods during one year.

SAMPLE NO. AND MID-DATE OF ITS BI-WEEKLY COL- LECTION PERIOD	PER CENT OF IODINE AS DETERMINED BY:		PER CENT MOISTURE LOST AT 97.5°	PER CENT IODINE CALCU- LATED TO DRY BASIS
	Seidell	Fenger		
1911				
1 September 1.....	0.528	0.56	0.55	0.531
2 September 16.....	0.434	0.46	1.20	0.440
3 October 1.....	0.416	0.45	0.50	0.418
4 October 14.....	0.411	0.44	3.83	0.428
5 October 28.....	0.381	0.41	0.90	0.385
6 November 11.....	0.273	0.30	11.34	0.308
7 November 25.....	0.318	0.34	7.74	0.344
8 December 10.....	0.305	0.33	6.99	0.328
9 December 24.....	0.337	0.34	7.00	0.362
1912				
10 January 7.....	0.275	0.29	7.70	0.298
11 January 21.....	0.165	0.19	8.40	0.180
12 February 4.....	0.173	0.18	8.95	0.190
13 February 18.....	0.219	0.21	2.23	0.224
14 March 3.....	0.147	0.16	8.15	0.160
15 March 17.....	0.125	0.12	3.76	0.133
16 March 31.....	0.188	0.20	1.07	0.190
17 April 14.....	0.232	0.23	8.46	0.254
18 April 28.....	0.255	0.26	1.37	0.259
19 May 12.....	0.244	0.25	8.78	0.268
20 May 26.....	0.285	0.28	10.82	0.320
21 June 9.....	0.294	0.31	10.28	0.328
22 June 23.....	0.344	0.35	11.92	0.391
23 July 7.....	0.341	0.34	11.49	0.385
24 July 21.....	0.301	0.31	10.21	0.335
25 August 4.....	0.398	0.39	13.97	0.463
26 August 18.....	0.381	0.41	11.01	0.428

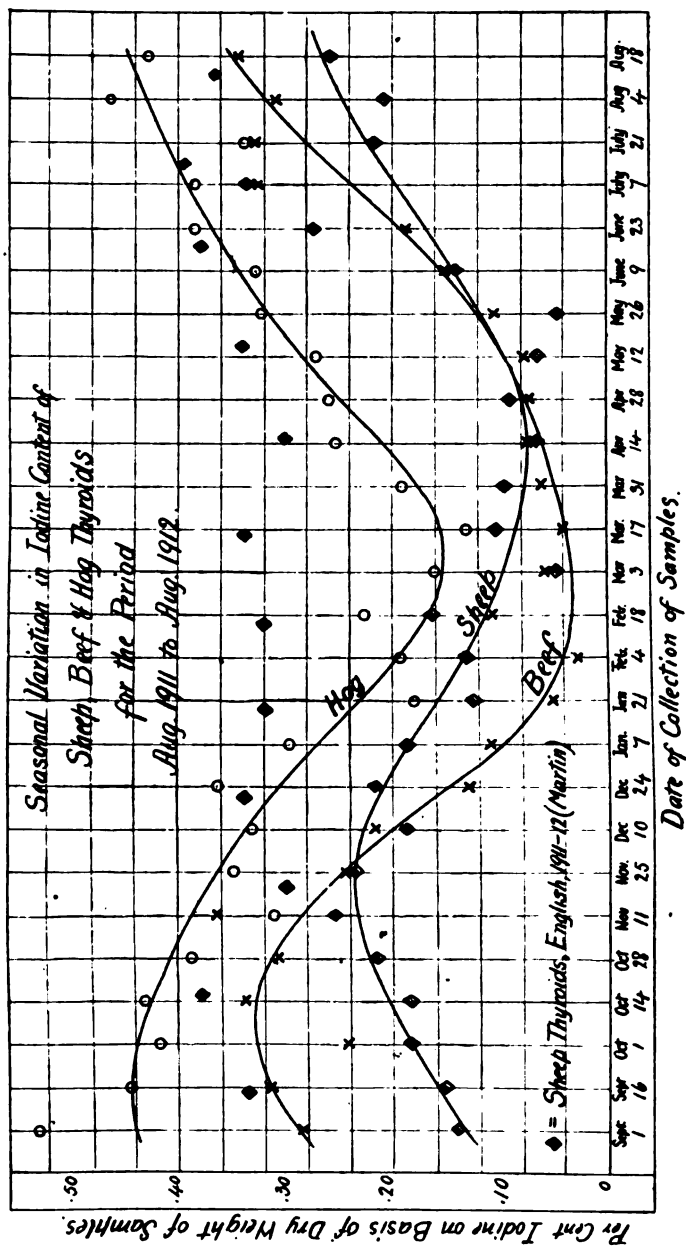
Before discussing the results of the iodine determinations in the samples, the following data upon the weight and size of the glands at the various seasons should be given. At the beginning of the experiment there were no particular reasons for suspecting a seasonal variation in the size of the gland, consequently the weights and number of glands in the several lots prepared for the iodine determinations were not taken. The averages here shown are therefore based upon figures obtained from time to time by counting out and weighing certain numbers of glands. These results are as follows:

ANIMAL	AVERAGE WEIGHT IN GRAMS PER SINGLE THYROID GLAND COLLECTED IN	
	Summer and Fall	Winter and Spring
Sheep.....	5.5	8.5
Beef.....	25.0	60.0
Hog.....	9.0	9.0

By comparison of the above results for sheep and beef glands with the diagram showing the seasonal variation in iodine content it will be seen that for the portion of the year during which the iodine content is lowest, namely, December to May, the size of the gland is greatest. It therefore follows that this seasonal change in iodine content and size of the glands accords with the frequently made observation that in general an inverse proportion exists between the size of the glands and the iodine content.

An examination of the results as recorded in the accompanying tables and diagram shows that, although fluctuations from a regular trend of seasonal change occur, these are small in comparison with the total differences in iodine content between the high and low season. Furthermore, the results show that the seasonal variation nearly coincides in the three species of animals studied. The average iodine content for the months of June to November is in general about three times that for the months of December to May. The maximum and minimum date for each of the three animals can not be selected with certainty, but in so far as can be judged from the diagram the high point for the hog comes first, that is early in September, for the beef it is early in October and for the sheep early in November. The fluctuations from the regularity of the curves are no doubt due largely to the individual variations in sex, age, diet, etc., among the animals, the thyroid glands of which form the composite sample in each case.

In a study of this kind it is manifestly impossible to follow the change in iodine content which takes place from month to month in the thyroid of an individual animal. The best that can be done is to use large numbers of animals and ascertain the average iodine content at a particular period of the year. This, however, involves averaging the very wide individual variations which are known to occur. Hence to overcome the effect of the individual variations it is necessary to use very large numbers of animals.



Results obtained in this way can at best show only a general average change which takes place, and the extent of the elimination of fluctuations will depend largely upon the number of glands represented by each composite sample. In the present experiment, in which 10 pounds of fresh glands were used for each sample, it appears that the divergence of the several points from the smooth curves, which were drawn as nearly as possible to represent the average of all the points in each series, is about as great in one case as in another. Since the total weight of glands was approximately the same for each sample, and the average weight of the individual glands from the three animals varied, the actual number of animals represented by each sample evidently differed considerably, being greatest with the sheep and least with the beef. This would therefore indicate that since the averages of a smaller number of beef glands showed no greater fluctuations than the averages of a larger number of sheep glands, the individual variation among sheep glands is probably somewhat greater than among the beef glands, but this point can of course not be definitely concluded.

For the sake of comparison the results communicated by Martin⁵ are also shown in the diagram and it will be noted that even with these there is a faint indication of a seasonal variation in harmony with the results upon American animals. In the case of the English sheep it is seen that the percentage of iodine during the whole year is much above that for the United States sheep even for the portion of the year during which it is highest. It may therefore be concluded that the geographical or climatic conditions which are the apparent cause of the high iodine content of the thyroid of English sheep are of sufficient intensity to overcome almost entirely the tendency towards the diminution of iodine content at the particular season of the year at which a lowered iodine content is shown by the present data to occur in the United States.

The determination of the moisture and ash in the present series of samples was made as follows. One gram of each sample was placed in a weighed porcelain crucible of about 15 cc. capacity, and the whole series of samples dried in a water bath at approxi-

⁵ *Loc. cit.*

mately 97.5°C. for fifteen hours. It was shown by separate experiments that even less than this length of time was sufficient to dry thyroid samples to constant weight. The loss in weight having been ascertained and calculated as moisture, the crucibles were placed, in groups of 10 each, in a small muffle furnace previously heated to redness. The complete incineration of the organic matter required about one half an hour.

In regard to the variations in the percentage of moisture as shown in the tables, it should be mentioned that during the preparation of the samples it became necessary to store quite a few of the sets in covered tin pails after extracting the fat and before powdering. This coarse and very porous material evidently reabsorbed moisture while standing in the pails and retained it during the grinding.

The individual ash determinations are not reported herewith since it was found that silicious material worn from the inner surface of the ball mill was present in the inorganic residues obtained. The proportion of ash varied with the time of grinding necessary to reduce the samples to powder of the requisite fineness. Analyses of it indicated the presence of more than 30 per cent of silica. The results therefore do not show the true ash content of the several thyroids but only the inorganic content of material prepared under particular conditions. In the case of the samples prepared between May 15 and July 15, it was necessary to discontinue the 6-hour day periods of grinding, and run the mill during the night for 15-hour periods. These samples are therefore averaged separately and as compared with the averages obtained upon samples ground in a Wedgewood mortar, and in a ball mill for six hours, show strikingly the effect of the longer period of grinding.

Percentage of ash.

	AVERAGE OF 8 SAMPLES GROUND IN WEDGEWOOD MORTAR	AVERAGE OF 13 SAMPLES GROUND IN PORCELAIN BALL MILL FOR 6 HOURS	AVERAGE OF 5 SAMPLES GROUND IN PORCELAIN BALL MILL FOR 15 HOURS OR LONGER
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Sheep thyroids.....	3.11	5.47	12.82
Beef thyroids.....	3.32	5.86	8.40
Hog thyroids.....	2.69	4.66	6.58

SUMMARY.

It has been shown that a very marked seasonal variation exists in the percentage of iodine present in the healthy normal sized thyroid glands of the sheep, beef and hog. There is in general about three times as much iodine present in the glands in the months between June and November as in the months between December and May.

A seasonal variation in the size of the fresh glands was observed in the case of the sheep and beef but not with the hog. The glands were found to be larger during the months in which the lower iodine content was observed.

The independent iodine determinations made upon this series of samples furnish gratifying evidence of the reliability of the Hunter method.

The maintenance of a standard of 0.2 per cent iodine in desiccated thyroid prepared for use in medicine will require the use of glands other than those of the sheep and, in addition, the mixing of the product obtained at the high and low seasons of the year.

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RESEARCHES ON PURINES.

ON 2,8-DIOXY-1,9-DIMETHYLPURINE AND 2-OXY-6,9-DIMETHYLPURINE.

(EIGHTH PAPER.¹)

By CARL O. JOHNS.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, December 18, 1912.)

Two of the nine possible isomerides of 2,8-dioxy-dimethylpurine have been described, namely, 2,8-dioxy-3,7-dimethylpurine,² which was obtained by Fischer, and 2,8-dioxy-6,9-dimethylpurine.³ The latter of these compounds, although isomeric with theobromine (VI), is not exactly analogous since one of the methyl groups is attached to carbon while in theobromine both of the methyl groups are attached to nitrogen. In 2,8-dioxy-1,9-dimethylpurine (IV) both of the methyl groups are attached to nitrogen, hence one might expect it to exhibit properties comparable with those of theobromine. Theobromine is a strong diuretic. Professor Mendel has tested the action of 2,8-dioxy-1,9-dimethylpurine on rabbits but found that it did not produce notable diuresis.⁴

2,8-Dioxy-1,9-dimethylpurine was synthesized as follows: The potassium salt of 2-oxy-5-nitro-6-methylaminopyrimidine⁵ (I) was heated with methyl iodide and gave 2-oxy-3-methyl-5-nitro-6-methylaminopyrimidine (II) the constitution of which was shown by heating the methylated product with sulphuric acid

¹ This *Journal*, xii, p. 91, 1912.

² Emil Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxviii, p. 2487, 1895; xxx, p. 1851, 1897; xxxii, p. 474, 1899.

³ Johns: this *Journal*, xi, p. 397, 1912.

⁴ Reported at the fourth annual meeting of the American Society for Pharmacology and Experimental Therapeutics, Cleveland, Dec. 30, 1912.

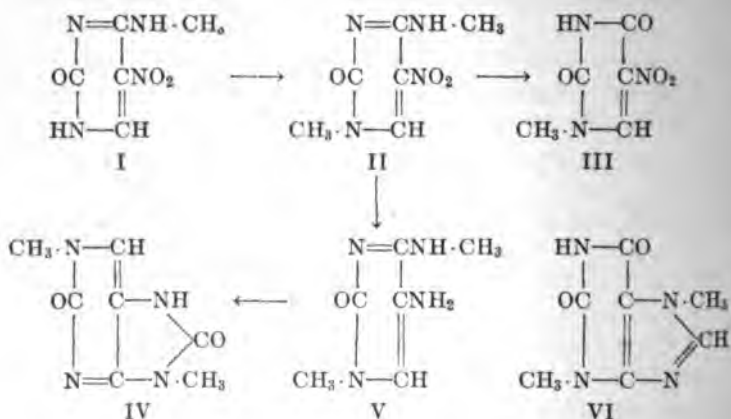
⁵ Johns: this *Journal*, ix, p. 164, 1911.

and obtaining 2,6-dioxy-3-methyl-5-nitropyrimidine⁶ (III). When 2-oxy-3-methyl-5-nitro-6-methylaminopyrimidine was reduced with freshly precipitated ferrous hydroxide it was converted to 2-oxy-3-methyl-5-amino-6-methylaminopyrimidine (V). As might be expected, the presence of the two methyl groups rendered the compound extremely soluble. It was therefore difficult to purify and after analyzing its picrate the crude base was heated with an equal weight of urea whereupon 2,8-dioxy-1,9-dimethylpurine (IV) was obtained.

A new isomer of the three 2-oxy-dimethylpurines⁷ previously described has also been prepared, namely, 2-oxy-6,9-dimethylpurine (IX). This compound was obtained by heating the potassium salt of acetyl-2-oxy-4-methyl-5,6-diaminopyrimidine (VIII).

During the course of this investigation it was found that 2-oxy-4-methyl-5,6-diaminopyrimidine can easily be produced in quantity by reducing 2-oxy-4-methyl-5-nitro-6-aminopyrimidine⁸ with freshly precipitated ferrous hydroxide. This method is a decided improvement on the one previously used in which aluminium amalgam was employed as the reducing agent.

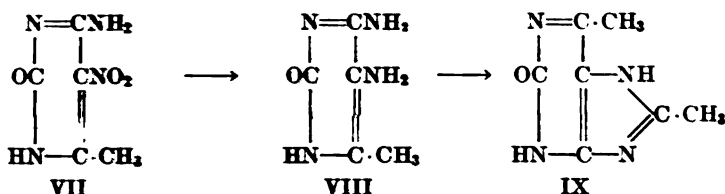
These researches will be continued.



⁶ Behrend and Thurm: *Ann. d. Chem.* (Liebig), cccxxiii, p. 163, 1902.

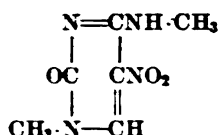
⁷ Julius Tafel: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 3201, 1899; Johns: *this Journal*, xii, p. 91.

⁸ Johns: *Amer. Chem. Journ.*, xli, p. 61, 1909.



EXPERIMENTAL PART.

2-Oxy-5-nitro-6-methylaminopyrimidine,

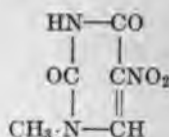


Five grams of finely pulverized 2-oxy-5-nitro-6-methylaminopyrimidine⁹ were dissolved in 40 cc. of water containing 1.9 grams of potassium hydroxide, and 5 grams of methyl iodide were then added. This mixture was heated at 100°C. in a sealed tube for one hour. This treatment produced an almost colorless solution from which a bulky mass of hair-like crystals separated on cooling. A second crop was obtained by concentrating the mother liquor. A trace of free iodine was removed by making the solution alkaline with sodium hydroxide. The crystals thus obtained were readily soluble in hot and moderately soluble in cold water. They were easily soluble in cold chloroform, moderately soluble in hot alcohol and slightly soluble in boiling benzene. They melted to a colorless oil at 203°C. The yield was about 50 per cent of theory.

	Calculated for C ₆ H ₆ O ₂ N ₄	Found:
N.....	30.43	30.12

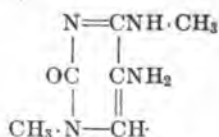
All of the nitropyrimidine used in the above experiment had reacted as none of it was recovered unaltered. An exceedingly soluble by-product was formed. This contained iodine that was not removed by treatment with alkalis. An investigation of this by-product was deferred until more material can be obtained.

⁹ Johns: this *Journal*, ix, p. 164, 1911.

2,6-Dioxy-3-methyl-5-nitropyrimidine,

In order to determine the position that the methyl group had occupied in the alkylation of 2-oxy-5-nitro-6-methylaminopyrimidine the methylamino group in position 6 was removed by hydrolysis as follows: One half gram of the methylated product was dissolved in 10 cc. of 25 per cent sulphuric acid and the solution was heated in a sealed tube at 160°–170° C. for two hours. On standing over night the resulting solution yielded a crop of beautiful crystals. These melted at 255° C. and when mixed with a pure sample of 2,6-dioxy-3-methyl-5-nitropyrimidine¹⁰ the melting point was not lowered. Hence alkylation had occurred in position 3 in the pyrimidine ring. The portion used for analysis was dried at 115° C. to remove the water of crystallization.

	Calculated for $\text{C}_5\text{H}_5\text{O}_4\text{N}_3$	Found:
N.....	24.56	24.55

2-Oxy-3-methyl-5-amino-6-methylaminopyrimidine,

Five grams of 2-oxy-3-methyl-5-nitro-6-methylaminopyrimidine were dissolved in 200 cc. of a mixture of equal parts of water and concentrated ammonia. Fifty grams of ferrous sulphate contained in 100 cc. of water were added. Reduction took place rapidly as was shown by the immediate formation of ferric hydroxide. A solution of 62 grams of crystallized barium hydroxide in hot water was then added to precipitate the SO_4 and a small excess of baryta was precipitated by means of ammonium carbonate. The mixture was heated on a steam bath for an hour and then

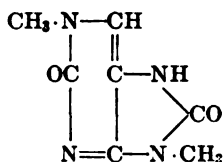
¹⁰ Behrend and Thurm: *loc. cit.*

filtered by suction. The filtrate was evaporated to a syrup which hardened on cooling. The substance thus obtained was exceedingly soluble in water or alcohol but did not dissolve in benzene or ether. On account of the difficulties encountered in attempting to purify this base it was used in the crude state for the preparation of 2,8-dioxy-1,9-dimethylpurine.

A picrate of 2-oxy-3-methyl-5-amino-6-methylaminopyrimidine was made by mixing a rather concentrated solution of the crude base with a saturated aqueous solution of picric acid. The picrate separated slowly in the form of prisms that were about five times as long as they were thick. They were very soluble in hot water. They melted with decomposition at about 200°C.

	Calculated for $C_8H_{10}ON_4 \cdot C_6H_3(NO_2)_3OH$	Found:
N.....	25.61	26.00

2,8-Dioxy-1,9-dimethylpurine,

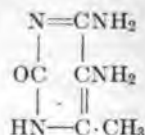


The crude 2-oxy-3-methyl-5-amino-6-methylaminopyrimidine which was obtained in the preceding experiment was mixed with an equal weight of urea and the mixture was heated for an hour at 180°C. in an oil bath. The residue was taken up in hot dilute ammonia and the solution was decolorized by means of blood coal. On boiling off most of the ammonia and acidifying with acetic acid the purine crystallized rapidly in the form of small plates. A second crop was obtained by concentrating the mother liquor to a small volume. The yield of pure material was about 50 per cent of theory when calculated on the basis of the nitropyrimidine used. The purine dissolved in less than 40 parts of boiling water and crystallized in small irregular plates that did not melt or char at 320°C. It dissolved readily in ammonia and was precipitated by acetic acid. It was slightly soluble in boiling alcohol but almost insoluble in boiling benzene. It did not give a murxide reaction.

0.1869 gram of substance gave 0.0740 gram of H_2O and 0.3174 gram of CO_2 .

	Calculated for $C_7H_8O_2N_4$	Found:
C.....	46.66	46.33
H.....	4.44	4.39
N.....	31.11	31.10

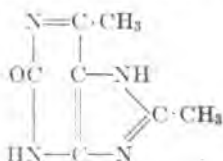
*2-Oxy-4-methyl-5, 6-diaminopyrimidine,*¹¹



This compound has been described in a previous communication from this laboratory. It was first obtained by reducing 2-oxy-4-methyl-5-nitro-6-aminopyrimidine by means of aluminium amalgam. This method was laborious as only small quantities could be reduced at one time and the yields were low. It has been found that larger quantities can be reduced smoothly by the use of freshly precipitated ferrous hydroxide.

Twenty-two grams of 2-oxy-4-methyl-5-nitro-6-aminopyrimidine were dissolved in a mixture of 200 cc. of water and 200 cc. of concentrated ammonia. To this solution were then added 264 grams of crystallized ferrous sulphate in a small volume of hot water. Reduction took place rapidly. The SO_4 was precipitated by adding a hot solution of 300 grams of crystallized barium hydroxide and removing the excess of baryta with ammonium carbonate. After filtering by suction, washing with hot water, and concentrating the filtrate the diaminopyrimidine was obtained in a crystalline form. The yield was 80 per cent of theory.

2-Oxy-6,8-dimethylpurine,



¹¹ Johns: *Amer. Chem. Journ.*, xli, p. 61, 1909.

Five grams of 2-oxy-4-methyl-5,6-diaminopyrimidine were suspended in 25 cc. of acetic anhydride and the mixture was heated in an evaporating dish on a steam bath. Complete solution did not occur as the crystals of the acetyl compound separated rapidly. The mixture was evaporated to dryness and the last traces of acetic anhydride were removed by adding a little alcohol and evaporating again and finally heating at 120° C. The yield agreed with that calculated for a diacetyl derivative. This substance was readily soluble in hot water and when the solution was cooled slowly a mixture of needles and rectangular prisms separated. The diacetyl compound was converted to a purine as follows:

Five grams of the acetylated 2-oxy-4-methyl-5,6-diaminopyrimidine were dissolved in 50 cc. of water containing 2.5 grams of potassium hydroxide and the solution was evaporated to dryness. The resulting potassium salt was then heated in an oil bath at 220°-240° C. until water was no longer evolved. The reaction product, which was apparently a potassium salt, was dissolved in hot water, the solution was filtered to remove a slight turbidity and was afterwards acidified with acetic acid. A crystalline precipitate formed rapidly. This was purified by dissolving it in dilute ammonia, clarifying with blood coal, boiling off excess of ammonia and precipitating with acetic acid. The crystals were small prisms with square ends. They did not melt at 315° C. although they turned brown slowly at that temperature. They were difficultly soluble in hot water, very slightly soluble in hot alcohol and insoluble in boiling benzene. They gave a murexide reaction. The yield of this purine was 70 per cent, calculated from the weight of 2-oxy-4-methyl-5,6-diaminopyrimidine.

0.2009 gram of substance gave 0.3758 gram of CO₂ and 0.0880 gram of H₂O.

	Calculated for C ₇ H ₈ ON ₂	Found:
C.....	51.21	51.02
H.....	4.87	4.86
N.....	34.14	34.26



THE CREATINE CONTENT OF MUSCLE UNDER NORMAL CONDITIONS. ITS RELATION TO THE URINARY CREATININE.¹

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(Received for publication, December 18, 1912.)

Although much attention has been devoted to urinary creatinine since the introduction of Folin's simple colorimetric method for its estimation in 1904, relatively little consideration has been given to the supposedly related muscle creatine. It is reasonable to believe that a knowledge of the creatine content of muscle, under normal and likewise abnormal conditions, may greatly aid in elucidating the various phases of creatine-creatinine metabolism. Attention has recently been called to some of these points by Mendel and Rose² who have cited the more important researches upon muscle creatine. van Hoogenhuyze and Verploegh³ were the first workers to employ the colorimetric method for the estimation of creatine in muscle, but to Mellanby⁴ is due the credit of first giving the subject any considerable attention. In addition, a few analyses have been reported by Dorner,⁵ Emmett and Grindley,⁶ Saiki,⁷ Graham-Brown and Cathcart,⁸ von Fürth and

¹ A preliminary report of this work was presented to the Society for Experimental Biology and Medicine, October 16, 1912; see *Proceedings*, x, p. 10, 1912.

² Mendel and Rose: this *Journal*, x, p. 255, 1911.

³ van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, xlvi, p. 433, 1905.

⁴ Mellanby: *Journ. of Physiol.*, xxxvi, p. 447, 1908.

⁵ Dorner: *Zeitschr. f. physiol. Chem.*, lii, p. 259, 1907.

⁶ Emmett and Grindley: this *Journal*, iii, p. 499, 1907.

⁷ Saiki: *ibid.*, iv, p. 487, 1908.

⁸ Graham-Brown and Cathcart: *Biochem. Journ.*, iv, p. 423, 1909.

Schwarz,⁹ and Okuda,¹⁰ most of these experiments being carried out with the idea of ascertaining conditions which might influence the creatine content of muscle. It does not appear to us, however, that our knowledge of the normal creatine concentration has been sufficiently complete to justify comparison with pathological variations. Very recently, Chisolm¹¹ has reported results for normal human muscle and compared these values with those found in malignant diseases and other pathological conditions.

The various results reported have served to indicate that animals of different species have a distinctive percentage content of muscle creatine, although the variations are small, from 0.3 to 0.5 per cent, and may be referred to, in general, as 0.4 per cent for all the higher vertebrates. Comparatively uniform figures have been obtained for a given animal by some of the above mentioned workers, by Mellanby for the frog, by Dorner and by Mellanby for the rabbit, likewise in the case of human muscle by Chisolm, who called attention to the comparatively uniform figures which he obtained. More analyses appear to have been reported for the rabbit than for any other animal, and the following figures for presumably normal rabbits may be cited:

Reported figures for the creatine content of rabbit muscle.

CREATINE IN FRESH MUSCLE	AVERAGES	OBSERVER*	CREATINE IN FRESH MUSCLE	AVERAGES	OBSERVER*
<i>per cent</i>			<i>per cent</i>		
0.529	0.506	Dorner	0.419	0.440	Graham-Brown and Cathcart
0.496			0.387		
0.496			0.477		
0.505			0.355		
			0.493		
	0.510	Mellanby	0.435		
			0.418		
			0.532		
0.520			0.449		
0.511			0.484	0.499	Mendel and Rose
0.409			0.528		
0.510			0.532		

* References previously cited.

⁹ von Fürth and Schwarz: *Biochem. Zeitschr.*, xxx, p. 413, 1911.

¹⁰ Okuda: *Eighth International Congress of Applied Chemistry, Original Communications*, xviii, p. 275, 1912.

¹¹ Chisolm: *Biochem. Journ.*, vi, p. 243, 1912.

In the instances in which these figures were given in terms of creatinine, they have been converted to creatine. With the exception of the results of Graham-Brown and Cathcart, the results are comparatively uniform. In their experiments, it should perhaps be noted that the figures were obtained with comparatively small samples of muscle (taken from one leg previous to the stimulation of the muscle on the opposite side).

Chisolm's results for the creatine content of the moist muscle of six healthy adults are as follows: 0.257, 0.290, 0.271, 0.280, 0.251 and 0.268 per cent, an average of 0.270.

Below is further appended the creatine content of muscle in a variety of animals as determined by various investigators with the colorimetric method, though in single experiments in many instances.

Reported figures (averages) for muscle creatine in man and various animals.

ANIMAL	CREATINE IN MUSCLE	OBSERVER*	ANIMAL	CREATINE IN MUSCLE	OBSERVER*
	<i>per cent</i>			<i>per cent</i>	
Rabbit..	0.51	Dorner	Horse ...	0.35	von Fürth and Schwarz
	0.51	Mellanby			
	0.44	Graham-	Dog.....	0.34	von Fürth and Schwarz
		Brown and Cathcart			
Pig.....	0.50	Mendel and Rose	Man.....	0.27	Chisolm
	0.45	van Hoogen-	Guinea pig.....	0.37	Mellanby
		huyze and Verploegh	Rat.....	0.35	Mellanby
	0.38	Mellanby	Hen.....	0.36	Mellanby
	0.44	van Hoogen-		0.41	Mendel and Rose
Ox.....		huyze and Verploegh	Frog.....	0.30	Mellanby
	0.44	Emmett and Grindley	Cod.....	0.35	Mellanby
		Mellanby	Lamphrey	0.29	Mellanby
	0.38	van Hoogen-	Skate.....	0.28	Mellanby
	0.41	huyze and Verploegh	Bonito.....	0.65	Okuda
Sheep...	0.38	van Hoogen-	Tunny fish.....	0.50	Okuda
		huyze and Verploegh	Salmon...	0.56	Okuda
	0.38	van Hoogen-	Snapper...	0.75	Okuda
Horse...		huyze and Verploegh	Carp.....	0.42	Okuda
			Shark.....	0.66	Okuda

* References previously cited.

In view of the lack of proof of the connection of creatine and creatinine in metabolism, our experiments were originally planned in an endeavor to ascertain whether in a given animal, in this case the rabbit, there was a constant relationship between the total creatine of the body and the daily urinary creatinine. In addition, this work was extended to include the determination of the creatine concentration of muscle of the rabbit and subsequently analyses were made upon the muscle of the dog, cat and, in a few instances, upon human muscle obtained at autopsies through the courtesy of Dr. O. S. Hillman.

Methods employed.

The rabbits used were well-fed animals of medium weight. The dogs and cats were the usual laboratory animals and appeared to be in good physical condition.

For extraction of creatine the various animals were killed by bleeding from the carotid. The skin was then removed from the hind legs, and the muscle, usually 100 grams, carefully freed from connective tissue and fat, ground in a small meat chopper and then mixed and covered with 95 per cent alcohol. In cases where the total creatine of the body was to be estimated (experiments with rabbits) the rest of the body was then skinned, viscera, except heart, removed and the remaining carcass passed through a large meat chopper, after which it was treated in a similar manner with 95 per cent alcohol. The samples were then preserved at 0°C. until the time of extraction, which was generally performed with little delay. Both the muscle sample and the carcass were subjected to repeated thorough extractions with water at temperatures from 20°–100°C. Seven extractions were usually made, the eighth extract being tested qualitatively for creatine. When an appreciable amount was present in this eighth extract, it was estimated and the proper correction made. The united extracts were assembled in an evaporating dish, evaporated down about one-third, slightly acidified with acetic acid to complete protein coagulation and filtered. The coagulum was extracted repeatedly until free from creatine. Usually two to three extractions sufficed for this. The extracts from the coagulum were added to the main filtrate, the whole evaporated to an appropriate volume, and finally made up to some definite volume

in a volumetric flask.¹² The carcass extracts were diluted to 1000 cc. and the muscle extracts usually to 250 cc. The extracts were then placed in perfectly clean and dry bottles and preserved with toluene.

Ten cubic centimeter samples of these extracts were taken in triplicates, treated with an equal amount of normal hydrochloric acid and the creatine converted to creatinine by the method suggested by one of us in collaboration with Benedict.¹³ The hydrolysis was carried out at 20 pounds pressure for twenty minutes. The method of extraction which we have employed yields an extract which is only slightly yellow,¹⁴ the color not being appreciably altered by this method of hydrolysis which appears particularly suitable in this connection. The creatinine thus formed was then estimated by the method of Folin in the usual way, except that ten minutes were allowed for the development of the color reaction.

The rabbits in which the content of body creatine was compared with the daily creatinine excretion were placed upon a uniform carrot diet for several preliminary days and then twenty-four hour urines collected daily for a period varying from five

¹² If an appreciable amount of coagulum was present, the solution was again filtered.

¹³ Benedict and Myers: *Amer. Journ. of Physiol.*, xviii, p. 395, 1907.

¹⁴ Various tests which we have made have given us considerable confidence in the procedures employed for the estimation of creatine. The method of extraction used by Mellanby (*Journ. of Physiol.*, xxxvi, p. 453, 1908) of evaporating the various aqueous extracts to dryness on the water bath and then taking up in 75 per cent alcohol to remove the creatine and creatinine, the alcohol subsequently being evaporated and an aqueous extract again prepared, appears to us to serve no useful purpose. In evaporating the extracts to dryness by this procedure, pigments are formed, though they probably do not interfere with the final estimation. Furthermore, the needless extraction with alcohol opens the way to possible error on account of the relatively low solubility of creatine in alcohol. Our extracts have been practically free from protein, though the presence of small amounts of protein does not interfere with the ultimate colorimetric readings. There is no reason to believe that the colorimetric method is not as accurate for the estimation of the creatine of muscle extracts (as creatinine) as the original method for the estimation of creatinine (and creatine) in urine. In fact, from some points of view it may be regarded as more accurate.

to fourteen days until it was believed that the correct daily creatinine elimination had been ascertained. Determinations were also made for creatine. At the end of the period, the animals were killed; body weights were taken daily previous to feeding, though the weights recorded in the table below were those taken just prior to the death of the animals.

Our results upon the creatine concentration of fresh muscle will be considered first.

Creatine content of rabbit muscle.

Table I gives the results which we have obtained from a series of twenty presumably normal rabbits.

TABLE I.

Creatine content of rabbit muscle.

ANIMAL NUMBER	BODY WEIGHT	WEIGHT OF MUSCLE SAMPLE	CREATINE IN FRESH MUSCLE
	<i>kgs.</i>	<i>grams</i>	<i>per cent</i>
1	1.48	100	0.522
2	1.55	100	0.540
3	1.49	100	0.522
4	1.77	100	0.522
5	1.39	100	0.522
6	2.12	100	0.494
9	2.05	100	0.497
10	2.13	{ 100	{ 0.524
		{ 100	{ 0.522
11	1.87	100	0.522
12	2.56	100	0.495
15	1.89	100	0.522
25	1.74	{ 75	{ 0.522
		{ 85	{ 0.527
27	1.35	100	0.517
28	1.90	100	0.522
29	1.85	100	0.521
30	1.60	100	0.534
31	1.80	100	0.522
32	1.66	100	0.495
33	2.05	100	0.522
34	1.98	100	0.516
Average content of creatine.			0.518

This series includes the animals made the subjects of our creatine-creatinine metabolism study and, in addition, a number of miscellaneous normal rabbits from which only muscle samples were obtained. In the first half dozen determinations, the colorimetric readings were first recorded without calculating our results. When this was done, we were surprised at the unusually uniform results. Our subsequent experiments entirely confirmed this. In fact, in going back over our notes of the various observations, incidents had been recorded which would possibly account for the slightly lower values in two cases. In animal 9, a correction of 5 mgms. had to be added for creatine found in the seventh extract, while an extract of the coagulated protein contained 12 mgms., indicating incomplete extraction. With animal 32, note was made that considerable difficulty was experienced in obtaining a good coagulation of the protein, though this fact was not recalled at the time the colorimetric estimation was made. Animal 12 was found to have a subcutaneous abscess. Even if we include these results, the creatine content of fresh rabbit muscle is found to be very definite and, as will appear below, characteristic of the animal.

In animal 10, the creatine contents of the left and right hind legs were found to be practically identical. In animal 25, the creatine content of the muscle of the right leg was found to be 0.522 per cent; that of the muscle of the back, adjacent to the vertebral column, 0.527 per cent. After allowing the left leg to remain at room temperature (very warm, July 20) for twenty-four hours, a creatine estimation of the muscle showed 0.482 per cent.

Creatine content of dog muscle.

In Table II are recorded five experiments on the creatine content of dog muscle. These figures are likewise very uniform, but considerably lower than in the case of rabbit muscle.

Creatine Content of Muscle

TABLE II.
Creatine content of dog muscle.

ANIMAL NUMBER	ESTIMATED WEIGHT	WEIGHT OF MUSCLE SAMPLE	CREATINE IN FRESH MUSCLE
	<i>kgs.</i>	<i>grams</i>	<i>per cent</i>
1	18	100	0.370
2	6	75	0.364
3	6	100	0.361
4	6	100	0.367
5	12	90	0.373
Average content of creatine.....			0.367

Creatine content of cat muscle.

The figures for the creatine content of cat muscle, as shown in Table III, are not as uniform as found in the case of the rabbit and dog, though, with two or three exceptions, the figures are fairly constant. Cat 3 was a starving animal, whose original weight was 3.19 kgs., and had been deprived of food for about forty days. Cat 11 was noted to be very poorly nourished. Cat 9 was a moderately fat pregnant animal. The low figure for Cat 16 cannot be accounted for. Although the variations are possibly connected with the nutritional state of the animal (fat or lean—ingestion of creatine with meat) we have no data to ex-

TABLE III.
Creatine content of cat muscle.

ANIMAL NUMBER	BODY WEIGHT	WEIGHT OF MUSCLE SAMPLE	CREATINE IN FRESH MUSCLE
	<i>kgs.</i>	<i>grams</i>	<i>per cent</i>
11	0.77	50	0.515
15	0.88	58	0.438
14	0.92	40	0.421
13	1.00	50	0.463
16	1.26	77	0.383
12	1.28	61	0.440
3	2.02	100	0.470
2	2.07	100	0.420
5	2.26	100	0.443
9	2.84	100	0.494
Average content of creatine.....			0.449

plain these variations. The weight does not appear to influence the content of creatine in the adult.

Influence of growth upon the creatine content of cat muscle.

In Table IV, figures are recorded for the muscle creatine of kittens from the same litter, but at different ages. These figures are in accord with the observations of Mellanby¹⁵ on rabbits and chickens of various ages.

TABLE IV.

Effect of growth on the creatine content of cat muscle.

ANIMAL NUMBER	WEIGHT OF ANIMAL	AGE	CREATINE IN FRESH MUSCLE
	<i>grams</i>	<i>weeks</i>	<i>per cent</i>
4	245	2	0.224
6	300	3	0.285
7	386	5	0.309
8	464	6	0.341
10	670	7	0.467

Creatine content of human muscle.

In two instances we have been able to obtain satisfactory samples of human muscle at autopsy with the following results.

The objection may be raised that the figures given for the muscle creatine of different animals are not comparable, because of the possibility of differences in composition, such as content of moisture or rather of solid matter. Estimation of moisture in supposedly

TABLE V.

Creatine content of human muscle.

SEX	CAUSE OF DEATH	SOURCE OF MUSCLE	CREATINE IN FRESH MUSCLE	MOISTURE OF MUSCLE	NITROGEN OF MUSCLE
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
F	Peritonitis	Abdominal	0.396	76.4	3.67
M	Amputation for sarcoma of the leg	Leg	0.391	76.3	3.44

¹⁵ *Loc. cit.*, p. 473.

normal samples of muscle has been made in only a few instances, because the figures were always found to be very uniform, not only for the same animal, but for animals of different species. The following figures for moisture, in addition to those for the human muscle above, may be cited: Dog 1, 75.5 per cent; Cat 5, 74.8 per cent; Cat 11, 75.8 per cent; Rabbit 12, 75.6 per cent; Rabbit 25, 74.5 per cent; Rabbit 35, 75.0 per cent.

Creatinine coefficient and muscle creatine.

It is a curious fact that the creatinine coefficient (milligrams of creatinine nitrogen eliminated per kilogram of body weight) of the rabbit is fully one-third higher than that found in man and the various experimental animals, dog, pig,¹⁶ cow¹⁷ and guinea pig.¹⁸ In forty rabbits in which we have had occasion to accurately determine the creatinine-nitrogen coefficient, it has been found to average 14.3. When the creatinine coefficients of the rabbit, dog and man are compared with the percentage content of muscle creatine in the same species, as shown in Table VI, an interesting and, as we believe, more than an accidental relationship is revealed. If creatinine has its origin in muscle creatine, or some common precursor substance, we would naturally expect from the creatinine coefficient of the rabbit to find that the muscle of this animal had a relatively high percentage content of creatine.

Although our figures given in Table VI for human muscle only represent two observations and are considerably higher than those given by Chisolm, we are not inclined to accept the figures of Chisolm as the normal, for even in children and in certain pathological conditions,¹⁹ we have failed to obtain results as low as those

¹⁶ Figures calculated from data of McCollum: *Amer. Journ. of Physiol.*, xxix, p. 210, 1911.

¹⁷ Figures calculated from data reported by Hart, McCollum, Steenbock and Humphrey: The University of Wisconsin Agricultural Experiment Station, Research Bulletin 17, June 1911.

¹⁸ Myers and Fine: Unpublished data.

¹⁹ In a baby 3 months old, weighing 3.2 kgms., and dying from cerebrospinal meningitis, muscle from the back and groin contained 0.321 per cent creatine. Muscle taken from the thigh in a case of marked edema in a man, gave 0.311 per cent of creatine and 80.5 per cent moisture. Muscle taken from the abdominal wall in a woman dying from general peritonitis,

TABLE VI.

Relation between muscle creatine and creatinine coefficient.

ANIMAL	NUMBER OF EXPERIMENTS	CONTENT OF CREATINE IN MUSCLE	NUMBER OF EXPERIMENTS	CREATININE COEFFICIENT
		<i>per cent</i>		
Rabbit....	20 normal animals	0.52	40 normal animals	14.3
Man.....	2 experiments	0.39	3 normal men about 27 years of age	9.0
Dog.....	5 normal animals	0.37	3 normal animals	8.4

reported by this author for supposedly normal muscle. We hope to further extend our studies on human muscle. According to Shaffer³⁰ the normal creatinine coefficient in man falls between 7 and 11. In three normal well-developed men about twenty-seven years of age, figures of 8.8, 9.0 and 9.2 respectively were obtained.²¹

The average of the figures which we have tabulated above for the creatine content of dog muscle probably represents the correct value, judging from the uniformity of the results upon which this figure was based. The creatinine coefficient in the dog shows some little variation, though the figure tabulated above probably approximates the average result. Shaffer²² states in this connection, "The kreatinin coefficients of normal dogs are practically the same as those given above for man. Wolf and Osterberg found an average of 8.2 for one dog and 7.0 for another. Results of my own from four dogs lie between the above figures."

contained 0.322 per cent creatine and had a moisture content of 80.2 per cent. The differences in the moisture content of the muscle in the last two instances, as compared with the figures tabulated above, practically accounts for the difference in the creatine results, *i.e.*, by reducing these last results to the tabulated water content, figures of about 0.384 are obtained.

³⁰ Shaffer: *Amer. Journ. of Physiol.*, xxiii, p. 1, 1908.

²¹ Figures calculated from experiments on W. W. H., V. C. M. and J. F. L. reported by Mendel and Myers: *ibid.*, xxvi, p. 77, 1910, and Mendel and Hilditch: *ibid.*, xxvii, p. 1, 1910.

²² *Loc. cit.*, p. 5.

The slight difference in the creatine content of dog muscle and human muscle, and in the creatinine coefficients of the same species (this statement could probably be made with regard to the pig, ox and guinea pig) is very interesting when compared with the uniformly greater values for the muscle creatine and the creatinine coefficient in the rabbit. The rabbit appears to be a unique animal in this respect. The comparatively high creatinine coefficient and high muscle creatine of the rabbit is very suggestive of the origin of creatinine from creatine.

With the view of ascertaining further data bearing on this point, experiments were planned in an endeavor to determine whether in a given animal, such as the rabbit, there was a constant relationship between the total creatine of the body and the daily creatinine. This ratio was ascertained in a series of one growing and eleven adult rabbits. The protocols follow.

Rabbit 1, a gray-brown male animal, was placed under observation on January 4, 1911, after a preliminary carrot diet period. The creatinine elimination was determined on five successive days as 19.6, 19.8, 19.4, 20.0 and 20.8 mgms. creatinine N, an average of 19.9 mgms., giving a coefficient of 13.4. Creatine was absent from the urine. On the morning of January 11, the animal weighed 1.48 kgms., previous to eating and at 10 a.m. was killed by bleeding after ether anesthesia. The total skinned and eviscerated carcass plus heart weighed 804 grams. The viscera minus heart weighed 392 grams. The carcass contained 2.633 grams creatine and the viscera, 0.082 gram.

Rabbit 2, a brown-gray male rabbit, after the usual preliminary uniform carrot diet, was placed under observation on January 4, and the creatinine elimination determined on seven successive days to be 23.5, 22.6, 20.3, 27.0, 21.5, 23.5 and 21.2 mgms. creatinine N, an average of 22.8 mgms. This figure gave a coefficient of 14.7 calculated from its body weight of 1.55 kgms., a weight which the animal maintained throughout the experiment. The rabbit eliminated no creatine. When killed on January 15, the skinned and eviscerated carcass plus the heart weighed 830 grams and contained 3.013 grams creatine. The viscera minus the heart weighed 460 grams and contained 0.069 gram creatine.

Rabbit 3, a female albino rabbit, after the customary preliminary diet was placed under study on January 9. The seven daily creatinine nitrogens were 24.7, 22.6, 23.4, 22.3, 19.9, 21.2 and 22.3 mgms., with an average of 22.3, giving a creatinine coefficient of 15.0 with the body weight of 1.49 kgms. obtained on the morning of January 18, without food, previous to being killed. No creatine was eliminated in the urine. The skinned and eviscerated carcass plus heart weighed 878 grams and contained 3.069 grams of creatine.

Rabbit 4, a female albino, was placed under observation in the usual way on January 9. The creatinine N elimination for five days was 24.1, 23.5, 24.7, 23.1 and 22.3 mgms., an average of 23.6 mgms., giving a creatinine coefficient of 13.4 with the body weight of 1.77 kgms. The urine contained no creatine. The animal was killed on January 23. The skinned and eviscerated carcass plus the heart weighed 940 grams and contained 3.096 grams of creatine.

Rabbit 5, a black male, was studied in the usual way from February 1-6. The creatinine N elimination per day for five days was 20.1, 18.4, 19.4, 20.5 and 19.8 mgms., an average of 19.6 mgms. The body weight was 1.39 kgms., giving a coefficient of 14.1. The animal eliminated no creatine. The skinned and eviscerated carcass plus the heart weighed 790 grams and contained 2.814 grams creatine.

Rabbit 6, a female albino of 2.12 kgms. weight, eliminated from January 9-14, daily, 32.2, 29.1, 29.1, 27.0 and 28.7 mgms. creatinine N, averaging 29.2 mgms. The creatinine coefficient was 13.7; no creatinine was eliminated. The animal was killed on January 29, the skinned and eviscerated carcass plus the heart weighing 1172 grams. It contained 3.535 grams creatine.

Rabbit 7, was a growing female albino, which showed a gain in weight from 0.59 to 0.69 kgm. during two weeks of observation, after beginning the experiment on February 24. The daily creatinine N elimination for 11 days was 6.9, 7.0, 6.5, 7.1, 7.3, 7.3, 7.1, 7.1, 8.0, 7.2 and 7.2, averaging 7.2 mgms., and giving a coefficient of 10.4 with the body weight of 0.69 kgms. There was a daily elimination of between 1 and 2 mgms. of creatine N.²² The weight of the skinned, eviscerated carcass employed for analysis was 290 grams and this contained 0.947 gram of creatine.

Rabbit 9, a light-brown female adult animal, weighing 2.05 kgms, eliminated daily the following amounts of creatinine N: 30.1, 30.6, 31.7, 29.1, 25.1, 25.1, 27.8, 25.1, 28.7, 27.6, 28.7, 27.0, 27.4, 27.4, 28.7, 28.7, 28.7, 29.3, 29.1 and 29.6 mgms., an average of 28.3 mgms., giving a coefficient of 13.8. Traces of creatine were detected in the urine on several days. The animal was killed on March 4 and the skinned and eviscerated carcass plus heart weighed 1140 grams and contained 3.385 grams of creatine.

Rabbit 10, a dark-gray female, weighing 2.13 kgms., eliminated daily 32.8, 32.9, 32.4, 34.5, 39.2, 32.4, 31.9 and 32.1 mgms. of creatinine N, averaging 32.3 mgms. This gave a coefficient of 15.2. The urine contained no creatine. The animal was killed on February 21 and the skinned and eviscerated carcass plus the heart weighed 1185 grams and contained 3.899 grams of creatine.

Rabbit 11, an albino female, after the usual preliminary diet was found to eliminate 27.0, 25.8, 25.8, 24.7, 26.6, 25.8, 25.1, 23.5, 24.5, 24.8, 26.0 and

²² The urine of growing animals contains creatine for some as yet unexplained reason. Cf. Closson: *Amer. Journ. of Physiol.*, xvi, p. 252, 1906; Rose: *this Journal*, x, p. 265, 1911; Folin and Denis: *ibid.*, xi, p. 253, 1912. We have also been able to verify this observation on children in some unpublished results.

24.8 mgms. of creatinine N per day, averaging 25.4 mgms. and giving a coefficient of 13.5 with its body weight of 1.87 kgms. No creatine was eliminated in the urine. The animal was killed on February 28; the skinned, eviscerated carcass weighed 965 grams and contained 3.070 grams creatine.

Rabbit 12, a gray female, was placed under observation in the usual way on March 18. The daily creatinine N elimination until March 29 was 37.9, 39.0, 35.1, 36.3, 30.1, 38.3, 38.3, 38.3, 37.4, 32.9 and 34.0 mgms. On April 9 this animal gave birth to three young. From April 11 to 16 the daily creatinine N was 38.3, 38.3, 40.5, 37.6 and 40.5 mgms., an average of 37.1 mgms. for both periods. This gave a creatinine coefficient of 14.5 with the body weight of 2.56 kgms. No creatine was detected in the urine. On April 16 the animal was killed. A subcutaneous abscess, weighing about 50 grams, was found in the skin on the ventral surface of the body. The skinned eviscerated carcass weighed 1480 grams and contained a moderate amount of fat, which was removed prior to extraction. The total amount of creatine was 4.317 grams.

Rabbit 15 was an albino female. Eight embryos were found when the animal was killed on March 23. The daily creatinine N elimination was 27.4, 27.0, 31.0, 28.5 and 27.0 mgms., averaging 28.2 mgms. This gave a coefficient of 15.2 when computed from the body weight of 1.89 kgms. The urine contained no creatine. The weight of the skinned, eviscerated carcass was 1035 grams and this contained 3.370 grams of creatine.

In the above protocols, the samples of muscle taken for creatine analysis have not been considered, as they do not appear to be concerned in the problem at hand, these special data having been recorded in Table I. Any creatine present in the internal organs or blood has not been tabulated, because it was thought that these small amounts, probably not over 0.1 gram in any case, would not invalidate our deductions.

In Table VII are given our results for this series of experiments. An inspection of this table shows that in the first five animals of nearly the same weight, about 2 kgms. (exclusive of the first rabbit, No. 12, weighing 2.56), the ratio between the total body creatine and the daily creatinine, was very constant, averaging 44.7 : 1. This included animals with high creatinine coefficients (15.3) and low coefficients (13.5). In other words, animals with a high creatinine elimination had a proportionately high body content of creatine. In rabbit 12, weighing 2.56 kgms., the creatine-creatinine ratio was 43.2 : 1. With smaller animals, the ratio of the creatine to the creatinine was found increased, e.g., in rabbit 5, weighing 1.39 kgms., it was observed to be 53.3 : 1. In the growing rabbit 7, which had a low creatinine coefficient, was eliminat-

TABLE VII.

Relation between total creatine of tissues and creatinine of urine in the rabbit. Data tabulated in order of body weight.

NUMBER OF ANIMAL	BODY WEIGHT AT DEATH	WEIGHT OF CARCASS	TOTAL BODY CREATINE	AVERAGE DAILY URINARY CREATININE	RATIO OF BODY CREATINE TO DAILY CREATININE	AVERAGE DAILY CREATININE NITROGEN	CREATININE COEFFICIENT	CONTENT OF BODY CREATINE
	kgms.	grams	grams	mgms.		mgms.	mgms.	per cent
12	2.56	1480	4.317	99.9	43.2	37.1	14.5	0.169
10	2.13	1185	3.899	86.9	44.9	32.3	15.2	0.183
6	2.12	1172	3.535	78.6	45.0	29.2	13.7	0.167
9	2.05	1140	3.385	76.2	44.4	28.3	13.8	0.165
15	1.89	1035	3.370	75.9	44.4	28.2	15.3	0.178
11	1.87	965	3.070	68.4	44.9	25.4	13.5	0.164
4	1.77	940	3.096	63.5	48.8	23.6	13.4	0.176
2	1.55	830	3.013	61.4	49.1	22.8	14.7	0.195
3	1.49	878	3.069	60.0	51.1	22.3	15.0	0.206
1	1.48	804	2.633	53.5	49.2	19.9	13.4	0.178
5	1.39	790	2.814	52.8	53.3	19.6	14.1	0.202
7	0.69	280	0.947	19.2	49.3	7.1	10.4	0.138

ing creatine and had a low percentage of body creatine, the ratio of total creatine to creatinine was 49.3 : 1, a figure not essentially different from the other rabbits next in size. It is possible that the slightly high ratios observed in the last six animals may all be explained on the basis of age. We appreciate the desirability of further data on this point. The ratio between the total body creatine and daily creatinine would appear to be the most reliable means of comparing these two supposedly related substances, because the factor of body weight is here eliminated from the calculations.

Nevertheless in Table VIII, the data from ten rabbits, exclusive of Nos. 12 and 7, the two extremes, have been arranged in order of the creatinine coefficients. The animals with a low percentage content of body creatine are seen to have low creatinine coefficients and *vice versa*. It is not believed that these figures are simply referable to variations in body weight which enter into these calculations. We endeavored to make our body weight figures as reliable as possible, the weight being taken in the morning prior to giving food and after compression of the bladder.

TABLE VIII.

Relation between per cent of body creatine and creatinine coefficient. Data tabulated in order of creatinine coefficients.

NUMBER OF ANIMAL	BODY WEIGHT	CREATININE COEFFICIENT	CONTENT OF CREATINE IN BODY	NUMBER OF ANIMAL	BODY WEIGHT	CREATININE COEFFICIENT	CONTENT OF CREATINE IN BODY
	kgms.		per cent		kgms.		per cent
4	1.77	13.4	0.176	5	1.39	14.1	0.202
1	1.48	13.4	0.178	2	1.55	14.7	0.195
11	1.87	13.5	0.164	3	1.49	15.0	0.205
6	2.12	13.7	0.167	10	2.13	15.2	0.183
9	2.05	13.8	0.165	15	1.89	15.3	0.178
Averages.....		13.6	0.170	Averages.....		15.0	0.193

In this way a period of twelve to fourteen hours generally elapsed after the taking of food. An inspection of Table VII shows that the creatinine elimination follows in every case the figures for body weight, which is an indication of the reliability of these figures.

This uniform relation between the creatinine elimination and the body weight, falls in line with the original statement of Folin²⁴ that "The chief factor determining the amount of creatinine eliminated appears to be the weight of the person," a point subsequently discussed by Benedict and one of us,²⁵ and by Shaffer.²⁶ The reason in this case is, in our opinion, clear. The relative proportion of the body tissues in a series of rabbits will be found relatively constant, *i.e.*, the adipose tissue is always small, in consequence of which the muscular tissue assumes a very uniform relation to the rest of the body. To this uniform proportion of muscle with its constant content of creatine, we are inclined to ascribe the constant relation between body weight and urinary creatinine. Shaffer's statement that "Kreatinin . . . is an index of some special process of normal metabolism taking place largely, if not wholly, in the muscles," appears in a new and added light.

²⁴ Folin: *Amer. Journ. of Physiol.*, xiii, p. 85, 1905.

²⁵ Benedict and Myers: *ibid.*, xviii, p. 392, 1907.

²⁶ Shaffer: *ibid.*, xxiii, p. 4, 1908.

Bearing in mind the constancy in the content of creatine in the striated muscle of the rabbit, it would appear possible, from our figures for total body creatine or per cent of body creatine (Table VII), to calculate the total amount of striated muscle in the body of the rabbit and its relative proportion to the body weight. Taking the average figure for the content of body creatine, about 0.182 per cent, 35 per cent of the weight of the rabbit would be in the form of muscle tissue. Should it be found possible to ascertain the factor influencing the relation between the total body creatine and the urinary creatinine, the amount of muscle tissue could be calculated from the daily creatinine elimination.

In a series of experiments (18) upon starving rabbits, which will be considered in a subsequent paper and have already been reported in abstract,¹⁷ it has been found that the creatine appearing in the urine in this condition accounts very largely for the creatine which has been lost from the muscle tissue. In a short fast this urinary creatine almost entirely accounts for the creatine lost by the tissues, but with an increase in the length of the fast this is progressively less true. This deficit we are inclined to ascribe to the creatinine eliminated during the period of starvation. Should this be the case, it would greatly strengthen the idea of the metabolic origin of creatinine from creatine or some common precursor substance. There are many factors which need to be considered in this connection such as specific enzymes, the oxidative power of the body toward these two compounds, etc. Experiments are in progress to determine the fate of administered creatine and creatinine, with methods similar to those outlined above.

CONCLUSIONS.

It has been pointed out that *the creatine content of muscle* in two typical animals, viz., the rabbit (herbivorous) and the dog (carnivorous) *is very constant*. For the cat the figures were somewhat less constant. Not only is the content of muscle creatine relatively constant for a given animal, but the figures *appear to be distinctive*, 0.52 per cent for the rabbit, 0.45 per cent for the cat, 0.37 per cent for the dog, 0.39 per cent for man, etc.

¹⁷ Myers and Fine: *Proc. Soc. for Exp. Biol. and Med.*, x, p. 12. 1912.

Another fact to which we have called attention and which we believe to be of particular interest in this connection, is the creatinine coefficients of the same animals. *The creatinine elimination appears to bear a distinct relation to the percentage content of muscle creatine in a given species.* The creatinine coefficient of the rabbit averages fully a third more than that of the dog and man, and we find similar relations with regard to the muscle creatine.

The constancy in the content of muscle creatine offers a satisfactory explanation for the constancy in the daily elimination of creatinine, first noted by Folin and subsequently confirmed by many workers. We have repeatedly observed that the daily creatinine in the rabbit may show almost identical figures for periods of days.

In rabbits of a nearly uniform weight it has been shown that *a constant relation exists between the total creatine of the body and the daily creatinine elimination.* Animals with a high creatinine coefficient appear to have a proportionately higher content of body creatine.

In our series of twelve rabbits, *the figures for the urinary creatinine follow the figures for body weight in every case.* The reason for this is, in our opinion, the uniform muscular development of the rabbit—very little adipose tissue—resulting in a relatively uniform body content of creatine, i.e., of creatinine-forming substance.

Based upon our figures for the creatine content of rabbit muscle and of the creatine concentration of the body, we have calculated that *striated muscle forms 35 per cent of the body weight in this animal.*

A NEW METHOD FOR DRYING TISSUES AND FLUIDS.

By JACOB ROSENBLOOM.

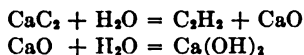
(From the Laboratory of Biochemistry of the University of Pittsburgh,
Pittsburgh, Pa.)

(Received for publication, December 27, 1912.)

The methods in use at present for drying tissues and fluids may be classified as follows: (1) Drying by means of salts; anhydrous sodium or calcium sulphate; (2) drying by means of alcohol; (3) drying in air or neutral gas.

As is well known all of these methods have many disadvantages. The methods based on drying by means of anhydrous salts often cannot be used because the mass obtained is inconveniently large when one expects to carry out a Soxhlet extraction on the material.

The method to be described is based on the reaction between calcium carbide and water:



and has many advantages, especially when drying tissues preliminary to the extraction of the contained lipins.

It may be noted that most of the water undergoes reaction with the carbide to form acetylene, the mass of material being much less on that account than when using calcium sulphate or sodium sulphate, because the water is taken up by these salts to form the hydrated salt and the water still remains in the material. Also the calcium oxide formed in the reaction is a dehydrating agent and facilitates the drying.¹ Another advantage in the calcium carbide method is that on account of the hardness of the carbide it is unnecessary to add sand in the mixing and grinding of the material.

¹ The presence of calcium oxide is especially advantageous when drying bile before extracting lipins as the calcium oxide forms an insoluble compound with the bile pigments and prevents their extraction by the solvents used.

Also after the substance is dried, on treatment with any of the lipin solvents it takes on a granular form, thereby giving the solvent the best chance of reaching the soluble lipins enclosed in the large amount of insoluble substance making up the tissue.

This method may be used without any precaution at all when one simply wants to extract *qualitatively* substances from tissues or fluids or for class-room work, such as the preparation of cholesterol from brain. However, when using it for drying material before using the material for *quantitative* analysis, I have so far only found one disadvantage. Owing to the fact that the reaction between calcium carbide and water is markedly exothermic, it is possible that the heat developed might decompose certain of the constituents. This can be minimized by adding the carbide in small amounts to the material to be dried, contained in a porcelain dish packed in ice.

When using the carbide for drying fluids, one simply adds the carbide in lumps until the material has lost its liquid form, then the powdered carbide is added till the drying is complete. The same method is used for tissues, with the addition of thoroughly mixing the carbide by grinding just as in the other methods.

On account of the fact that acetylene is explosive and poisonous it is wise to carry out the drying under a hood, free from flames.

In regard to any possible change that the acetylene might produce in the chemical nature of the constituents of the tissues or fluids dried by this method I can say that no differences could be detected in the character of the lipins of heart muscle (ox) and of urine, when obtained from material dried by this method, when compared with that obtained from heart muscle and urine, dried by means of anhydrous calcium sulphate.

PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND TISSUE ANALYSIS.

SIXTH PAPER.

ON URIC ACID, UREA AND TOTAL NON-PROTEIN NITROGEN IN HUMAN BLOOD.

BY OTTO FOLIN AND W. DENIS.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, December 31, 1912.)

In earlier papers of this series we have reported studies on the non-protein nitrogenous products of the blood from the standpoint of absorption. The definite nature of the results recorded was largely due to the fact that the new technique employed proved suitable for the work. In the absence of unusually rapid absorption or of abnormally high levels of protein destruction (as in fevers) the non-protein nitrogen of the blood must be a more or less sharp index to the efficiency of the kidneys in removing the waste nitrogenous products circulating in the blood. The investigation of the non-protein nitrogenous constituents of human blood is especially interesting from this point of view because of its possible clinical bearings.

We are well aware, of course, that a great many investigations have already been made in this field; in fact, ever since the time of Bright, clinicians have been interested in the "retention nitrogen" of the blood and have tried hard to connect it in one way or another with "uremia."¹ But on the whole the results have not proved particularly convincing, instructive or helpful, either to physiologists or to clinicians. By means of our new methods, however, we expected at least to be able to measure various degrees of nitrogen retention and urea accumulation due to kidney inefficiency with much greater accuracy than had heretofore been possible.

¹ For a résumé of much of the literature on this subject see Obermayer and Popper: *Zeitschr. f. klin. Med.*, pp. 72, 332, 1911.

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... of this constituent in blood is,
... of the earlier investigators realized,
... The amount in normal blood is,
... out of reach by titration meth-
... investigations as to the extent to which

... human blood is concerned we have
... with the theoretical deduction
... because it is rather abundant in the
... in the blood in cases of gout, leukemia
... demonstrated by a number of different
... claimed long ago to have demon-
... in normal human blood as well as
... from gout or nephritis.³ But there
... Garrod drew to a considerable extent
... the "microscopic identification,"
... peroxide reaction for the demonstration
... The modern precipitation methods (the
... of Krüger) have proved inadequate for
... in normal human blood. Magnus-Levy,
... and Schittenhelm, all have failed
... blood by the help of these methods.
... obtained positive results. But at all
... acid in normal human blood has been
... entirely impossible, and a "positive uric
... extensively used and generally accepted as
... diagnosis of gout.⁴

... our paper on a method for determining
... is as a matter of fact enough uric acid
... to make its quantitative determination
... as simple as the determination of the
... protein nitrogen.

³ 1912.

⁴ of Gout.

... Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, iv,
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All three determinations can be made without requiring more than 20 to 30 cubic centimeters of blood. We have therefore in a combination of these three determinations practically a new system of blood analysis—certainly one well worth trying out on human blood for clinical purposes.

As a preliminary to such work and having for the first time a method capable of detecting extremely minute amounts of uric acid we thought it worth while also to make a general survey of the blood of different kinds of animals. The results of this survey are given in table 1.

TABLE 1.

Uric acid, total non-protein nitrogen and urea nitrogen in blood.

(The figures represent milligrams per 100 grams of blood.)

	URIC ACID	NON-PROTEIN NITROGEN	UREA NITROGEN
Rabbit (6 cases).....	0.05	31	13
Sheep (mixed blood).....	0.05	28	13
Pig (mixed blood).....	0.05	32	14
Horse (1 case, antitoxin animal)...	0.05	54	28
Monkey (1 case polyomyelitis).....	0.05	60	38
Beef (mixed blood).....	0.2	24	14
Cat (2 cases: diet, liver).....	0.2	60	34
Cat (2 cases: diet, milk and eggs) ..	0.2	67	37
Cat (2 cases: diet, rice and cream) ..	0.2	31	20
Chicken (6 cases, mixed blood).....	4.9	32	8
Duck (4 cases, mixed blood).....	4.8	34	7
Goose (1 case).....	4.8	26	8

From the results obtained it would appear that the uric acid in the blood of rabbit, sheep and horse is almost nil, and that in the case of the other animals the amount though a trifle larger is still extraordinarily small—0.2 of a milligram or less per 100 grams of blood. In bird's blood where the origin of the uric acid is entirely different from that of mammals the amount is, of course, very much larger, but still is not as large as we had expected to find it. It might not be out of place in this connection to call attention to the small amounts of urea found in the blood of birds as compared with the amounts occurring in blood of mammals.

The results of our first survey of the uric acid in human blood are given in table 2. The figures can scarcely be said to represent the strictly normal variations for no attempt was made to select physically normal persons. All the samples of blood for the analyses recorded in this table were obtained from newly admitted patients at the Massachusetts Psychopathic Hospital in Boston.*

The uric acid figures of table 2 are arranged according to the amount of uric acid found. We have also made an arbitrary division of the figures into three groups. We have done this only to facilitate inspection with regard to the amount of uric acid most frequently found in human blood. The uric acid figures

TABLE 2.

Uric acid in unselected human blood.

(The figures represent milligrams per 100 grams of blood.)

Group 1, 5 cases.....	{	0.7	0.8	0.8	0.9
		0.9			
	{	1.0	1.0	1.2	1.2
		1.2	1.3	1.4	1.4
Group 2, 22 cases.....	{	1.4	1.5	1.5	1.5
		1.5	1.6	1.6	1.6
		1.6	1.6	1.6	1.8
	{	2.0	2.0		
	{	2.2	2.4	2.5	2.6
Group 3, 11 cases.....		2.8	2.8	2.8	2.9
		3.0	3.5	3.7	

recorded in group 2 representing from 1 to 2 milligrams of uric acid per 100 grams of blood are we believe well within the normal variations, but we are not prepared to say that they represent the full variations.

It is a curious and interesting fact that human blood contains several times as much uric acid as does the blood of any other mammal whose blood we have had the opportunity to examine. The exceptional position of man indicated by the high uric acid and extremely little allantoin in urine, as recently referred to by

* The blood was obtained in connection with the application of the "Wasserman reaction" and we wish to express our obligation to Dr. H. Adler for having given us a large number of samples.

Wiechowski⁷ and by Hunter and Givens,⁸ is thus again sharply indicated by the uric acid content of the blood.

For the urea and total non-protein nitrogen of human blood we do have figures representing strictly normal material. The blood was obtained from our medical students and from instructors in the department. It was drawn in the morning 3-6 hours after breakfasts which varied from "tea and toast" to "one egg, one pork chop, cereal, bread and cocoa."

TABLE 3.

Total non-protein nitrogen and urea nitrogen in normal human blood.

(Figures indicate milligrams per 100 grams of blood.)

NAME	AGE	NON-PROTEIN NITROGEN	UREA NITROGEN
O.F.....	45	26	13
C.F.....	26	25	12
H.L.....	35	26	13
C.E.....	35	25	13
J.D.....	26	23	12
M.D.....	28	25	11
H.T.....	24	24	11
L.R.....	22	26	11
A.G.....	22	26	12
H.B.....	22	22	11
L.W.....	20	26	13
C.S.....	25	22	11
E.C.....	22	23	12
S.E.....	22	25	12
J.M.....	22	26	12
A.S.....	23	24	13

From the figures for non-protein nitrogen and urea nitrogen in table 3 it will be seen that the former varies only between 22 and 26 milligrams and the latter only between 11 and 13 milligrams per 100 grams of blood. The narrowness of the range of variation of these figures is quite remarkable. It would seem that the perfectly normal kidney maintains a remarkably constant level of non-protein nitrogen and urea nitrogen in the blood. A "perfectly normal kidney" appears, however, to be the exception rather than the rule just as soon as one leaves the thoroughly

⁷ *Biochem. Zeitschr.*, xxv, p. 433, 1910.

⁸ *This Journal*, xiii, p. 372, 1912.

healthy adults and begins to work on any kind of "clinical material." The contrast between the normal non-protein nitrogen and urea figures of table 3 and the corresponding values obtained from syphilitics, as recorded in table 4, is rather appalling. Out of the 63 cases cited, and these were taken in the order of the examinations without any selection, there are but 13 whose values are not higher than the normal as represented in table 3. The

TABLE 4.

Non-protein nitrogen and urea nitrogen in the blood of syphilis patients.
(Figures are given in milligrams per 100 grams.)

NON-PROTEIN NITROGEN	UREA NITROGEN	NON-PROTEIN NITROGEN	UREA NITROGEN	NON-PROTEIN NITROGEN	UREA NITROGEN
41	23	43	25	28	13
34	22	41	23	34	22
31	18	36	22	30	20
31	18	33	20	37	16
34	22	33	20	38	16
34	20	22	12	36	20
35	23	31	15	39	18
32	18	27	14	36	17
34	21	20	10	44	26
31	18	25	12	45	21
30	15	26	13	42	18
36	20	25	13	35	17
32	13	24	12	36	19
31	17	33	20	39	19
36	20	25	12	41	17
41	23	25	12	42	15
34	21	23	11	42	16
38	25	33	20	43	17
35	21	30	15	41	15
38	21	27	14	32	16
41	21	25	12	42	22

ages of these patients varied from fourteen to about seventy, the time of infection from a few weeks to about twenty years.⁹

That the impaired efficiency of the kidney indicated by the figures recorded in table 4 is not peculiar to syphilis is shown by corresponding values obtained from the insane. Out of 21 of these, the figures for which are given in table 5, there are only

⁹ We are indebted to Dr. Abner Post for this blood.

7-10 whose nitrogen and urea figures are normal when judged by the standard values of table 3.

In view of the striking difference in the condition of the blood of patients at hospitals and clinics as compared with that of strictly normal adults we were eager to see what kind of values would be obtained from patients suffering from recognized nephritis. The results obtained from eleven cases of "chronic nephritis" are given in table 6. Of these not a single one gave normal urea or non-protein nitrogen figures. The lowest figure obtained from nephritic blood is half again as large as the highest obtained from normal blood. On the other hand some of the nephritic patients

TABLE 5.

Non-protein nitrogen, urea nitrogen and uric acid in unselected human blood.

(The figures represent milligrams per 100 grams.)

NON-PROTEIN NITROGEN	UREA NITROGEN	URIC ACID	NON-PROTEIN NITROGEN	UREA NITROGEN	URIC ACID
43	18	1.2	32	17	2.4
27	14	1.4	24	13	2.0
24	18	1.3	35	18	2.6
50	22	1.2	62	46	2.1
40	21	3.0	36	19	2.8
52	32	1.6	28	16	2.0
24	13	2.0	32*	18	2.5
50	26	1.4	36	19	1.7
36	19	0.8	28	16	2.4
28	16	2.0	32	19	1.8
34	18	1.5			

carry no greater accumulation of waste nitrogen in the blood than that found in some of the patients afflicted with syphilis or insanity. In how many of the latter it might have been possible for an expert clinician to make a diagnosis of chronic nephritis cannot now be determined.¹⁰

In the nitrogen and urea records of table 6 it will be noted that in the blood of one patient (No. 5, A. S.) the figures are nearly twice as large as the corresponding figures of any other in the table and nearly four times as large as the normal.

¹⁰ This paper represents only a preliminary survey of the field—a survey which we hope to follow up later by more exhaustive studies.

TABLE 6.

Uric acid, urea nitrogen and non-protein nitrogen in nephritic blood.

(Figures represent milligrams per 100 grams.)

DESCRIPTION OF CASE	URIC ACID	NON-PROTEIN NITROGEN	UREA NITROGEN
1. M. P., "old lady"; poorly nourished; blood pressure (sys.), 200. Urine: blood cells, casts.	1.2	50	22
2. T. F., age 65; "chronic nephritis," sys. blood pressure, 220. Oedema, dyspnoea. Urine: albumin, casts and blood.....	2.7	40	19
2. Same patient one week later.....	2.5	38	19
3. J. D., age 63, "chronic nephritis," arterio-sclerosis, aneurism aorta, very cyanotic.....	2.5	54	21
4. E. H. T., age 68, blood-pressure 260, no cardiac symptoms. Urine: trace of albumin, hyaline and granular casts.....	3.9	5.2	20
5. A. S., age 45; blood-pressure 170, no oedema, skin very dark and variable in color. Urine: albumin, casts, blood.....	2.2	93	68
6. Ch. W., age 57; rheumatic for years; no oedema, no dyspnoea. Urine: albumin, hyaline and granular casts.....	1.1	42	21
7. J. F., age 55; oedema in the feet; dyspnoea. Urine: albumin, pus, no casts.....	2.1	41	21
8. Ch. M., age 46; alcoholic, oedema in the feet, dyspnoea. Urine: hyaline and granular casts and blood cells.....	1.0	43	29
9. O. D., age 38; "fairly acute nephritis", headache, vomiting. Urine: albumin, hyaline, granular and waxy casts.....	2.5	46	19
10 R., age 57; no cardiac symptoms, slight oedema; blood-pressure 180. Urine: trace albumin, casts. "Early stage of interstitial nephritis".....	2.0	46	19
11. M. L., age 51; blood-pressure 160. Urine: casts, much albumin and pus. Carcinoma of the breast and chronic nephritis—Dr. Ordway.....	2.9	50	33

In connection with an extensive series of investigations of human blood in various diseases, but particularly nephritis, Strauss subdivided the cases studied into chronic, interstitial and chronic parenchymatous nephritis (with and without uremia). Strauss worked with blood serum, taking 100-200 cc. of blood from each patient, and precipitated the proteins by means of acetic acid. It appears to us very improbable that the procedure employed by Strauss for removing the proteins could give uniform results. At least that is our experience in connection with the determination of uric acid in blood when we use a very similar method for removing the proteins. Traces of albuminous materials make no difference in our uric acid results, but would of course completely destroy the value of nitrogen determinations such as Strauss made. Strauss' results are nevertheless decidedly interesting. But his conclusion that the non-protein nitrogen in chronic interstitial nephritis averages 82 mgm. per 100 cc. of serum and 40 mgm. in parenchymatous nephritis¹¹ is certainly not verified by our results reported in table 6.

The figures recorded in tables 5 and 6 are noteworthy in that they probably represent the first analyses on record where the uric acid, the urea and the total non-protein nitrogen have been determined in the same samples of human blood. *Further, from the figures we learn at once that there is apparently no relationship between the amount of uric acid and the amount of urea or total non-protein nitrogen in human blood.*

The above italicized generalization is, we think, of fundamental importance from a clinical as well as from a physiological point of view. The urea and total non-protein nitrogen in the blood must in the main be inversely proportional to the general efficiency of the kidney since the kidney represents practically the only outlet for the nitrogenous waste products. The significance of the uric acid is less clear. In the absence of some other plausible explanation, it, too, should occur in the blood in amounts inversely proportional to the ability of the kidney to remove it. Such a view was accordingly in earlier times used to explain gout (Garrod), a disease characterized by the accumulation of uric acid in the blood.

This simple explanation, naturally the first one to be thought of, has not been able to withstand the criticisms to which it has been submitted. To clinicians it has seemed incorrect because gout and general kidney inefficiency do not go hand in hand.

¹¹ *Die chronischen Nierenentzündungen*, 1902, p. 24.

From a physiological standpoint it was far-fetched because it assumed a specificity of the kidney toward different waste products which was not warranted by anything that was known concerning the secretion of urine.

A more modern explanation of the absence of any proportion between the uric acid and the other nitrogenous waste products in blood would be borrowed from the current teachings with regard to the destruction of uric acid within the body. Given some region, some tissue or organ, capable of destroying uric acid, and partial stagnation due to kidney inefficiency would result in the circulation of the uric acid laden blood over and over again through such a region and the accumulation of uric acid would be prevented. A few years ago no one would have hesitated to accept this last explanation. The work of Burian and Schur seemed to furnish a substantial experimental basis for it. Just now there is room for a certain amount of hesitancy because of the genetic relationship which has been discovered to exist between uric acid and allantoin in the urine (Wiechowski).

Allantoin is regarded as a final waste product whose precursor is uric acid. The urine of animals which contains little or no uric acid contains instead allantoin in amounts sufficient to account for the missing uric acid. In man, however, the allantoin excretion is almost nothing, amounting only to 10 or 15 milligrams or even less per twenty-four hours. The practical absence of allantoin in human urine has at once raised the issue whether uric acid is destroyed within the human organism. Wiechowski, Sivén and Hunter and Givens¹² have taken the position that uric acid is probably a final waste product in man and is not destroyed by any human tissue. Frank and Schittenhelm adopt the older view of Burian and Schur that the destruction of uric acid within the human organism is quite extensive; and Brugsch and Schittenhelm¹³ have formulated a theory of gout based on a variable fermentative destruction of uric acid, the failure of which in gout is due to a defective cell metabolism and results in the accumulation of uric acid in the blood.

¹² For the recent literature on the subject see the article by Hunter and Givens already cited.

¹³ *Loc. cit.*

If the amount of uric acid in human blood were always small, i.e., at the normal level, compared with the accumulation of the other nitrogenous waste products, there would hardly be room for any differences of opinion as to the ability of the human organism to decompose uric acid, notwithstanding the fact that it, unlike other animal organisms, does not convert the uric acid into allantoin. The situation is, however, rendered more complex by the well established fact that in certain kinds of human blood (gout and lead poisoning) there is an *accumulation* of uric acid.

Analyses of human blood characterized by a high uric acid content are recorded in table 7. For the time being we venture to call this blood "uric acid blood" in order to emphasize our finding that it is abnormally rich in uric acid without containing correspondingly large amounts of urea or other waste nitrogen. The distinctive character of this blood is we think unmistakable. The uric acid may reach the saturation point (in the form of urate of soda) and may be as abundant as in the blood of birds!

There is still a certain amount of dispute as to whether the uric acid elimination in gout is or is not appreciably diminished.

No one, however, claims that the uric acid output is increased above that found in the normal. This is important. The mere fact that the uric acid may accumulate in the blood of the gouty without being accompanied by an increased elimination constitutes definite proof that the gouty kidney is damaged with reference to its ability to eliminate uric acid.

The hypothesis that there is normally a fermentative destruction of uric acid within the human tissues and that this destructive process is diminished in gout, as suggested by Brugsch and Schittenhelm, is hopelessly inadequate because the undisputed fact is that the gouty kidney fails to respond with an increased elimination of uric acid when there are noteworthy accumulations of uric acid in the blood. Further, the fact that some gouty kidneys are capable of eliminating the urea and other nitrogenous waste products almost or quite as well as strictly normal kidneys (see Nos. 1 and 6, table 7) comes very near proving that the kidney activity is as selective as is the secretory activity of any other gland. In pure gout unaccompanied by any abnormal urea retention in the blood the kidney is damaged (so far as we yet know)

The ammonia in blood has received a large amount of attention in the study of nephritis. The determination of this constituent in blood is, however, far more difficult than any of the earlier investigators realized, and their results are probably worthless. The amount in normal blood is, as we now know, so minute as to be entirely out of reach by titration methods.² We have not yet made any investigations as to the extent to which it may be found in pathological blood.

So far as the uric acid in human blood is concerned we have heretofore had to remain satisfied with the theoretical deduction that it must always be there because it is rather abundant in the urine and because its presence in the blood in cases of gout, leukemia and lead poisoning has been demonstrated by a number of different investigators. To be sure, Garrod claimed long ago to have demonstrated the presence of uric acid in normal human blood as well as in the blood of those suffering from gout or nephritis.³ But there is room for the suspicion that Garrod drew to a considerable extent on his imagination when applying the "microscopic identification," the "thread test" and the murexide reaction for the demonstration of uric acid in blood. The modern precipitation methods (the methods of Salkowski and of Krüger) have proved inadequate for the task of finding uric acid in normal human blood. Magnus-Levy, Klemperer, von Jaksch, Brugsch and Schittenhelm, all have failed to find the uric acid in normal blood by the help of these methods. A few, notably Petrén, have obtained positive results. But at all events the detection of uric acid in normal human blood has been extremely uncertain, if not entirely impossible, and a "positive uric acid test" is accordingly extensively used and generally accepted as a valuable aid in the diagnosis of gout.⁴

As already indicated in our paper on a method for determining uric acid in blood⁵ there is as a matter of fact enough uric acid in normal human blood to make its quantitative determination (by that method) almost as simple as the determination of the urea or the total non-protein nitrogen.

² This *Journal*, xi, p. 167, 1912.

³ *The Nature and Treatment of Gout*.

⁴ See Brugsch and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, iv, pp. 438, 446, 480, 532, 538, 551, 1907. See also Gudzent: *Deutsch. med. Wochenschr.*, xxxviii, p. 603, 1912.

⁵ See the last number of this *Journal*, p. 469.

All three determinations can be made without requiring more than 20 to 30 cubic centimeters of blood. We have therefore in a combination of these three determinations practically a new system of blood analysis—certainly one well worth trying out on human blood for clinical purposes.

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The results of our first survey of the uric acid in human blood are given in table 2. The figures can scarcely be said to represent the strictly normal variations for no attempt was made to select physically normal persons. All the samples of blood for the analyses recorded in this table were obtained from newly admitted patients at the Massachusetts Psychopathic Hospital in Boston.*

The uric acid figures of table 2 are arranged according to the amount of uric acid found. We have also made an arbitrary division of the figures into three groups. We have done this only to facilitate inspection with regard to the amount of uric acid most frequently found in human blood. The uric acid figures

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		0.9			
	{	1.0	1.0	1.2	1.2
		1.2	1.3	1.4	1.4
Group 2, 22 cases.....	{	1.4	1.5	1.5	1.5
		1.5	1.6	1.6	1.6
		1.6	1.6	1.6	1.8
	{	2.0	2.0		
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34	22	41	23	34	22
31	18	36	22	30	20
31	18	33	20	37	16
34	22	33	20	38	16
34	20	22	12	36	20
35	23	31	15	39	18
32	18	27	14	36	17
34	21	20	10	44	26
31	18	25	12	45	21
30	15	26	13	42	18
36	20	25	13	35	17
32	13	24	12	36	19
31	17	33	20	39	19
36	20	25	12	41	17
41	23	25	12	42	15
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24	18	1.3	35	18	2.6
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52	32	1.6	28	16	2.0
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50	26	1.4	36	19	1.7
36	19	0.8	28	16	2.4
28	16	2.0	32	19	1.8
34	18	1.5			

carry no greater accumulation of waste nitrogen in the blood than that found in some of the patients afflicted with syphilis or insanity. In how many of the latter it might have been possible for an expert clinician to make a diagnosis of chronic nephritis cannot now be determined.¹⁰

In the nitrogen and urea records of table 6 it will be noted that in the blood of one patient (No. 5, A. S.) the figures are nearly twice as large as the corresponding figures of any other in the table and nearly four times as large as the normal.

¹⁰ This paper represents only a preliminary survey of the field—a survey which we hope to follow up later by more exhaustive studies.

TABLE 5.

Uric acid, urea nitrogen and non-protein nitrogen in nephritic blood.

(Figures represent milligrams per 100 grams.)

DESCRIPTION OF CASE	URIC ACID	NON-PROTEIN NITROGEN	UREA NITROGEN
1. M. P., "old lady"; poorly nourished; blood pressure (sys.), 200. Urine: blood cells, casts.	1.2	50	22
2. T. F., age 65; "chronic nephritis;" sys. blood pressure, 220. Oedema, dyspnoea. Urine: albumin, casts and blood.....	2.7	40	19
2. Same patient one week later....	2.5	38	19
3. J. D., age 63, "chronic nephritis," arterio-sclerosis, aneurism aorta, very cyanotic.....	2.5	54	21
4. E. H. T., age 68, blood-pressure 260, no cardiac symptoms. Urine: trace of albumin, hyaline and granular casts.....	3.9	5.2	20
5. A. S., age 45; blood-pressure 170, no oedema, skin very dark and variable in color. Urine: albumin, casts, blood.....	2.2	93	68
6. Ch. W., age 57; rheumatic for years; no oedema, no dyspnoea. Urine: albumin, hyaline and granular casts.....	1.1	42	21
7. J. F., age 55; oedema in the feet; dyspnoea. Urine: albumin, pus, no casts.....	2.1	41	21
8. Ch. M., age 46; alcoholic, oedema in the feet, dyspnoea. Urine: hyaline and granular casts and blood cells.....	1.0	43	20
9. O. D., age 38; "fairly acute nephritis", headache, vomiting. Urine: albumin, hyaline, granular and waxy casts.....	2.5	46	19
10 R., age 57; no cardiac symptoms, slight oedema; blood-pressure 180. Urine: trace albumin, casts. "Early stage of interstitial nephritis".....	2.0	46	19
11. M. L., age 51; blood-pressure 160. Urine: casts, much albumin and pus. Carcinoma of the breast and chronic nephritis—Dr. Ordway.....	2.9	50	33

In connection with an extensive series of investigations of human blood in various diseases, but particularly nephritis, Strauss subdivided the cases studied into chronic, interstitial and chronic parenchymatous nephritis (with and without uremia). Strauss worked with blood serum, taking 100-200 cc. of blood from each patient, and precipitated the proteins by means of acetic acid. It appears to us very improbable that the procedure employed by Strauss for removing the proteins could give uniform results. At least that is our experience in connection with the determination of uric acid in blood when we use a very similar method for removing the proteins. Traces of albuminous materials make no difference in our uric acid results, but would of course completely destroy the value of nitrogen determinations such as Strauss made. Strauss' results are nevertheless decidedly interesting. But his conclusion that the non-protein nitrogen in chronic interstitial nephritis averages 82 mgm. per 100 cc. of serum and 40 mgm. in parenchymatous nephritis¹¹ is certainly not verified by our results reported in table 6.

The figures recorded in tables 5 and 6 are noteworthy in that they probably represent the first analyses on record where the uric acid, the urea and the total non-protein nitrogen have been determined in the same samples of human blood. *Further, from the figures we learn at once that there is apparently no relationship between the amount of uric acid and the amount of urea or total non-protein nitrogen in human blood.*

The above italicized generalization is, we think, of fundamental importance from a clinical as well as from a physiological point of view. The urea and total non-protein nitrogen in the blood must in the main be inversely proportional to the general efficiency of the kidney since the kidney represents practically the only outlet for the nitrogenous waste products. The significance of the uric acid is less clear. In the absence of some other plausible explanation, it, too, should occur in the blood in amounts inversely proportional to the ability of the kidney to remove it. Such a view was accordingly in earlier times used to explain gout (Garrod), a disease characterized by the accumulation of uric acid in the blood.

This simple explanation, naturally the first one to be thought of, has not been able to withstand the criticisms to which it has been submitted. To clinicians it has seemed incorrect because gout and general kidney inefficiency do not go hand in hand.

¹¹ *Die chronischen Nierenentzündungen*, 1902, p. 24.

From a physiological standpoint it was far-fetched because it assumed a specificity of the kidney toward different waste products which was not warranted by anything that was known concerning the secretion of urine.

A more modern explanation of the absence of any proportion between the uric acid and the other nitrogenous waste products in blood would be borrowed from the current teachings with regard to the destruction of uric acid within the body. Given some region, some tissue or organ, capable of destroying uric acid, and partial stagnation due to kidney inefficiency would result in the circulation of the uric acid laden blood over and over again through such a region and the accumulation of uric acid would be prevented. A few years ago no one would have hesitated to accept this last explanation. The work of Burian and Schur seemed to furnish a substantial experimental basis for it. Just now there is room for a certain amount of hesitancy because of the genetic relationship which has been discovered to exist between uric acid and allantoin in the urine (Wiechowski).

Allantoin is regarded as a final waste product whose precursor is uric acid. The urine of animals which contains little or no uric acid contains instead allantoin in amounts sufficient to account for the missing uric acid. In man, however, the allantoin excretion is almost nothing, amounting only to 10 or 15 milligrams or even less per twenty-four hours. The practical absence of allantoin in human urine has at once raised the issue whether uric acid is destroyed within the human organism. Wiechowski, Sivéń and Hunter and Givens¹² have taken the position that uric acid is probably a final waste product in man and is not destroyed by any human tissue. Frank and Schittenhelm adopt the older view of Burian and Schur that the destruction of uric acid within the human organism is quite extensive; and Brugsch and Schittenhelm¹³ have formulated a theory of gout based on a variable fermentative destruction of uric acid, the failure of which in gout is due to a defective cell metabolism and results in the accumulation of uric acid in the blood.

¹² For the recent literature on the subject see the article by Hunter and Givens already cited.

¹³ *Loc. cit.*

If the amount of uric acid in human blood were always small, i.e., at the normal level, compared with the accumulation of the other nitrogenous waste products, there would hardly be room for any differences of opinion as to the ability of the human organism to decompose uric acid, notwithstanding the fact that it, unlike other animal organisms, does not convert the uric acid into allantoin. The situation is, however, rendered more complex by the well established fact that in certain kinds of human blood (gout and lead poisoning) there is an *accumulation* of uric acid.

Analyses of human blood characterized by a high uric acid content are recorded in table 7. For the time being we venture to call this blood "uric acid blood" in order to emphasize our finding that it is abnormally rich in uric acid without containing correspondingly large amounts of urea or other waste nitrogen. The distinctive character of this blood is we think unmistakable. The uric acid may reach the saturation point (in the form of urate of soda) and may be as abundant as in the blood of birds!

There is still a certain amount of dispute as to whether the uric acid elimination in gout is or is not appreciably diminished.

No one, however, claims that the uric acid output is increased above that found in the normal. This is important. The mere fact that the uric acid may accumulate in the blood of the gouty without being accompanied by an increased elimination constitutes definite proof that the gouty kidney is damaged with reference to its ability to eliminate uric acid.

The hypothesis that there is normally a fermentative destruction of uric acid within the human tissues and that this destructive process is diminished in gout, as suggested by Brugsch and Schittenhelm, is hopelessly inadequate because the undisputed fact is that the gouty kidney fails to respond with an increased elimination of uric acid when there are noteworthy accumulations of uric acid in the blood. Further, the fact that some gouty kidneys are capable of eliminating the urea and other nitrogenous waste products almost or quite as well as strictly normal kidneys (see Nos. 1 and 6, table 7) comes very near proving that the kidney activity is as selective as is the secretory activity of any other gland. In pure gout unaccompanied by any abnormal urea retention in the blood the kidney is damaged (so far as we yet know)

TABLE 7.

Uric acid, urea nitrogen and non-protein nitrogen in "uric acid blood."

(Figures represent milligrams per 100 grams.)

DESCRIPTION OF CASE.	URIC ACID	NON-PROTEIN NITROGEN	UREA NITROGEN
1. Dr. H., age 53. Gout. Several typical attacks during the past seven years. No tophi. Urine normal. Last attack about six months ago.....	3.9	25	13
2. Mr. B., age 65. "Gout and vascular nephritis."—Dr. Pratt...	5.5	52	36
3. Same case on a purine-free diet.....	3.4	40	18
4. Mr. T., age 29. Gout and alcoholic gastritis.—Dr. Pratt....	3.5	40	23
5. Mr. S., "Pure gout, normal, very healthy, tophi."—Dr. Pratt....	4.4	30	15
6. Mr. B., typical gout, last attack two years ago.—Dr. Joslin.....	5.2	28	13
7. Mr. L., age 37; leukemia. Spleen greatly enlarged. Abdomen skin pigmented. Oedema of feet.—Dr. Ordway.....	3.1	33	14
8. Same as 7 on a purine-free diet..	2.8	44	20
9. Mr. C., age 45; leukemia. Large spleen. Urine normal.—Dr. Ordway.....	4.1		
10. Mr. X., age 45, lead poison, third attack. Alcoholic for some years.....	4.7	50	31
11. Mr. Y., Lead poison.....	4.8	52	32
12. Mr. E., three attacks of pain in lumbar region during the past year, each time passed several small stones. No arthritis, no nephritis, no gout. X-ray showed no stones in kidney.—Dr. Larrabee. The stones contained over 95 per cent uric acid.	5.2	30	13

only with regard to its function of removing down to the normal level the uric acid of the blood.¹⁴

The damage to the kidney resulting in the retention of uric acid and in the development of gout may be a very slight damage. The normal kidney clearly does not remove the uric acid from the blood as completely as the negative uric acid findings of previous investigators seemed to indicate. Normal human blood contains several times as much uric acid as they thought. Gouty blood on the other hand seems to contain less.¹⁵ Normal blood contains not less than from 1 to 2 or 2.5 milligrams of uric acid per 100 grams of blood. The blood in gout does not exceed 6 milligrams at least so far as our present experience goes. There is, however, no reason to suppose that a uric acid concentration of 4 to 6 milligrams per 100 grams of blood is very much more irritating or stimulating to the kidney than the somewhat more dilute solutions represented by normal blood. Disregarding the insolubility of uric acid the elevation of its threshold of elimination from 2 to 4 or 6 milligrams (per 100 grams of blood) is certainly a small one. Kidneys in which the threshold of elimination for urea has risen by 10 or 20 milligrams (per 100 grams) or even more are as we have seen (tables 4 and 5) extraordinarily common.

Such urea (and total nitrogen) retentions may represent latent or incipient nephritis, but in many, perhaps in most of these, the nephritis must remain latent or incipient, otherwise cases of recognized nephritis would be much more common than they are. Whether any recognizable effects on health may be traced to such urea retentions remains to be determined. In the case of uric acid it seems to be purely a matter of chance, purely a matter of insolubility, that corresponding or even smaller degrees of kidney insufficiency and slight uric acid accumulation should result in all the serious consequences involved in the development of gout.

¹⁴ In leukemia (see Nos. 7, 8, 9, table 7) the cause of the high uric acid in the blood is an increased production, and the kidney responds by eliminating large quantities of uric acid.

¹⁵ From a consideration of the analytical technique employed by earlier investigators it is not difficult to see that they would entirely overlook moderate amounts of uric acid (1-2 mgm.) yet would get (by weight or by nitrogen determinations) entirely too much if they found any at all.

We have said nothing in this communication concerning endogenous versus exogenous uric acid in blood—a distinction strongly emphasized by Brugsch and Schittenhelm in their papers on gout. The distinction is one long considered from a therapeutic dietetic standpoint. It may prove somewhat important from the standpoint of diagnosis, as well, in doubtful cases. As in the case of the urea the difference between the normal and the abnormal uric acid level in the blood should however be unmistakable without regard to reasonable variations in the purine intake.

The investigations of the accumulation of waste products in human blood in various diseases are being continued.

THE CHEMISTRY OF GLUCONEOGENESIS.

II. THE FORMATION OF GLUCOSE FROM VALERIANIC AND HEPTYLIC ACIDS.

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In a previous communication¹ under this title, evidence was presented to the effect that in phlorhizinized animals, the administration of 10 grams of propionic acid, as ammonium or sodium salts, *per os* or subcutaneously, was followed by an increase in the glucose elimination which corresponded to all of the carbon of the propionic acid. The suggestion was then made that phlorhizinized animals have the power of quantitatively² synthesizing propionic acid into glucose. In this series of experiments the influence of the homologues of propionic acid are considered.

Methods.

Female dogs were used in all the experiments. The urine was collected by catheter, and the bladder washed with distilled water at the end of every period. Merck's phlorhizin was used, and was injected in 2-gram doses three times per twenty-four hours, as recommended by Lusk.³ The following methods were used in the analyses: nitrogen, Kjeldahl-Gunning; ammonia, Folin; total acetone, Huppert-Messinger; aceto-acetic acid, Embden; glucose, the Allihn gravimetric method, also by polarization after clarifying the urine with basic lead acetate; β -hydroxybutyric acid, determined by Magnus-Levy's method. The β -hydroxybutyric acid results are relative, not absolute, for it was found that a considerable amount of

¹ A. I. Ringer: The Chemistry of Gluconeogenesis. I. The Quantitative Conversion of Propionic Acid into Glucose, this *Journal*, xii, p. 511, 1912.

² By quantitative, in this case, we understand the utilization of the entire molecule for the synthesis of glucose.

³ Graham Lusk: Phlorhizinglukosurie, *Ergeb. d. Physiol.*, xiii, p. 315, 1912.

phlorhizin was eliminated by the kidneys in the urine. This phlorhizin is extracted with the ether and has a specific rotation of -42.3° . The result of this investigation will be reported in the near future.

The effect of formic acid (HCOOH).

In the first table are tabulated the results of an experiment in which the animal received 11.5 grams of formic acid, subcutaneously, as sodium salt dissolved in 50 cc. of water. There was no increase in the glucose, nor any increase in the D : N ratio. In experiment V, period V, the animal received a similar amount of formic acid, with similarly negative results. These two experiments show that *the diabetic organism does not possess the power of utilizing formic acid in the synthesis of glucose.*

The effect of normal butyric acid ($\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-COOH}$).

In experiment V, period III, the animal received subcutaneously, 10 grams of normal butyric acid (Merck) dissolved in 50 cc. of water and neutralized with sodium hydroxide. In experiment VI, period IV, the animal received 20 grams of the same butyric acid, administered in a similar manner. In neither experiment was there any increase in the glucose elimination. The aceto-acetic acid and β -hydroxybutyric acid, however, were increased quite markedly. During period V of experiment VI the animal was in deep coma, presenting all the typical symptoms of diabetic coma, and it ended in death.

The effect of normal valerianic acid ($\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-COOH}$).

In experiment VII, period II, 9.2 grams of normal valerianic acid (Kahlbaum), dissolved in 50 cc. of water and neutralized with sodium hydroxide, were administered subcutaneously. The glucose elimination, which was 19.23 grams in the first period, rose to 24.13, in spite of a lower nitrogen metabolism in that period. The D : N ratio, which normally has a tendency to sink with the progress of the glucosuria, rose from 3.38 in the first period to 5.05 and 3.97 in the second and third periods, respectively. It is evident therefore that "extra glucose" was eliminated during periods II and III which did not find its origin in the catabolized protein. As no analyses were made during period IV of that experiment, we are forced to assume that the D : N ratio in

periods II and III would have remained at 3.38 had no valerianic acid been given. In calculating the "extra glucose" an error is thus introduced which makes the results a little too low. The extra glucose can be calculated by the following formula: $EG = G - (N \times Q)$; E G stands for extra glucose, G stands for the value of the glucose eliminated, N stands for the value of the nitrogen, Q stands for the value of the assumed $D : N$ quotient.

G in periods II and III is equal to $24.13 + 18.54 = 42.67$ grams.

N in periods II and III is equal to $4.78 + 4.67 = 9.45$ grams.

Q in periods II and III is assumed to be 3.38.

$$EG = G - (N \times Q) = 42.67 - (9.45 \times 3.38) = 10.7.$$

9.2 grams of normal valerianic acid give rise to 10.7 grams of extra glucose.

In experiment IX, period II, the animal received 14.2 grams of normal valerianic acid administered as above. The glucose elimination rose from 18.28 grams in the fore period to 26.20 and 21.08 in the second and third periods respectively. The $D : N$ ratio, which was 3.55 in the fore period, rose to 5.28 and 3.89 in the second and third periods.

Calculation of "extra glucose."

G in periods II and III is equal to $26.20 + 21.08 = 47.28$ grams.

N in periods II and III is equal to $4.96 + 5.41 = 10.37$ grams.

Q in periods II and III is assumed to be $\frac{3.55 + 3.64}{2} = 3.6$.

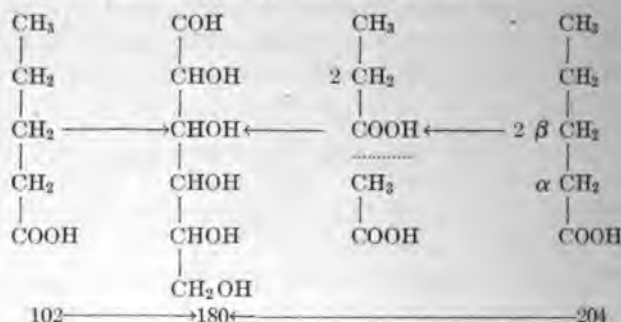
$$EG = G - (N \times Q) = 47.28 - (10.37 \times 3.6) = 9.95.$$

14.2 grams of normal valerianic acid give rise to 9.95 grams of extra glucose.

These two experiments agree in showing that normal valerianic acid can be utilized in the synthesis of glucose. Although the extent of the utilization differs widely in these two experiments, we are justified, from the very nature of experiments on phlorhizinized animals, in accepting the maximal figures as the ones which show the maximum extent of synthesis. In experiment VII we obtained 10.7 grams of glucose from 9.2 grams of normal valerianic acid. Calculated per 10.0 grams of valerianic acid, the yield of glucose is equal to 11.6 grams. In experiment IX, 14.2 grams of valerianic acid yielded 9.95 grams of glucose, which gives for 10.0 grams of this acid 7.0 grams of glucose. How can

46 Formation of Glucose from Fatty Acids

we assume this glucose to have arisen from the valerianic acid molecule? A glance at the relationship of the valerianic acid molecule to the glucose molecule may throw light on this.



There are two possible ways in which valerianic acid may be converted into glucose. The first is by the utilization of all the carbons of valerianic acid in the upbuilding of the glucose molecule. In this case, 10.2 grams of the acid should give rise to 18.0 grams of glucose. This is not in accord with the findings in the experiments, and is besides highly improbable. The other possibility is that the products of decomposition of valerianic acid are synthesized into glucose. From the researches of Knoop, Embden, Dakin⁴ and others, it is well established that the normal fatty acids in the animal body undergo oxidation and cleavage in the β-position. Valerianic acid would then give rise to propionic acid, which has already been shown by the writer⁵ to be completely converted into glucose. Two molecules of valerianic acid would therefore be required for the formation of one molecule of glucose. Expressed in gram-molecular weight 10.2 grams of valerianic acid should give rise to 9 grams of glucose, which is in accordance with our findings. The conclusion is therefore justified that *valerianic acid gives rise to glucose in so far as it can give rise to propionic acid.*

The effect of normal caproic acid (CH₃-CH₂-CH₂-CH₂-CH₂-COOH).

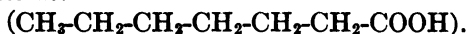
In experiment VIII, period II, the animal received 9.2 grams of normal caproic acid (Kahlbaum) as sodium salt. In experiment

⁴ H. D. Dakin: *Oxidations and Reductions in the Animal Body*, Longmans, Green and Company, 1912, contains the most recent review of the literature.

⁵ *Loc. cit.*

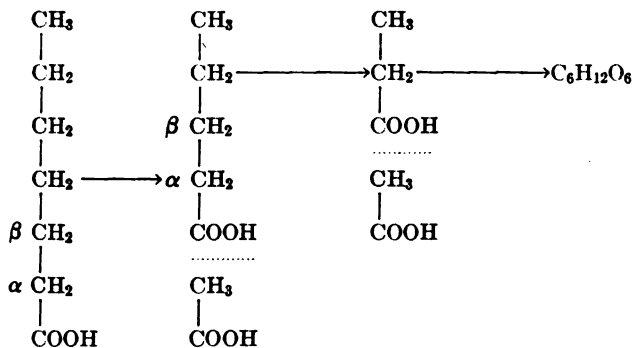
IX, period V, the animal received 10.3 grams of the same acid. In neither case was there any increase in the glucose elimination. The acetone bodies, aceto-acetic acid and β -hydroxybutyric acid were increased considerably, especially in experiment IX. These two experiments corroborate the findings of earlier investigators⁶ who found that caproic acid is a β -hydroxybutyric acid builder.

The effect of normal heptylic acid



In experiment IX, period VII, 13 grams of normal heptylic acid were administered subcutaneously. The glucose elimination in that period was increased, but to a lesser extent than after valerianic acid feeding. Assuming that all of the extra glucose came out in that period, then $E\ G = G - (N \times Q) = 19.15 - (4.13 \times 3.75) = 3.67$ grams of extra glucose. In experiment X, period II, 13 grams of heptylic acid were administered as above. The glucose rose from 15.63 in the fore period to 20.99; the D : N ratio rose from 3.71 to 4.20.

$E\ G = G - (N \times Q) = 20.99 - (5.0 \times 3.78) = 2.09$ grams of extra glucose. The amount of glucose derived from heptylic acid is indeed small, but an examination of the table convinces one that there is a decided increase, and that it can come from no other source. The heptylic acid no doubt undergoes oxidation and cleavage in the β -position, being converted into valerianic acid and finally into propionic acid, and this fraction of the heptylic acid molecule goes over into glucose.



⁶ H. D. Dakin: *loc. cit.*

The yield of glucose in these two experiments was not as great as might theoretically be expected. Further experiments will be performed soon, with the hope of establishing a more exact quantitative relationship.

It is very evident from these experiments that, while the fatty acids with the even number of carbons, as butyric and caproic acids, give rise to aceto-acetic acid and β -hydroxybutyric acid, those with an uneven number of carbons give rise to glucose. The amount of glucose that arises diminishes with the size of the fatty acid molecule, and there is every reason to believe that it is only the three final carbons which contribute to the glucose formation. If this is proven to be true in the case of the higher fatty acids with C_{15} and C_{17} , it may be of the utmost value in the treatment of diabetes mellitus.

It is now very well established that aceto-acetic acid and β -hydroxybutyric acid find their origin mainly in the catabolism of the fatty acids with an even number of carbon atoms. 256 grams of palmitic acid in severe cases of diabetes may give rise to as much as 104 grams of β -hydroxybutyric acid. Should we find that the fats containing C_{15} or C_{17} (which are in progress of preparation now) are burned in the body, we may thus have a very efficacious means of combating acidosis. From the above experiments we may rightly conclude that the fatty acids with an uneven number of carbons undergo oxidation in the β -position, and thus they *cannot* possibly give rise to acids with an even number of carbons.

Very interesting, also, is the fact that, in every case in which gluconeogenesis takes place, it is associated with a coincident antiketogenic process. It is illustrated very markedly in the case of the propionic acid feeding.⁷

Experiments on the different phases of this problem and their bearing on diabetes are being carried on, and I beg to be permitted to reserve this field of research for a reasonable length of time.

SUMMARY.

Experiments were performed on phlorhizinized dogs and the glucose, nitrogen, ammonia, acetone, aceto-acetic acid and β -hydroxybutyric acid eliminations were studied.

I. The administration of formic acid is not followed by any increase in the glucose elimination.

II. Butyric and caproic acids produce an increase in the aceto-acetic and β -hydroxybutyric acid eliminations, but no increase in the glucose.

III. Valerianic and heptylic acids give rise to glucose, in all probability because of the formation of propionic acid as an intermediary body in their catabolism, after undergoing β -oxidation.

IV. Attention is called to the fact that the fatty acids with an uneven number of carbon atoms give rise to glucose.

The experimental data will be found in the following tables.

Dec.

EXPERIMENT IV. *Twelve-hour periods.*

DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D:N	REMARKS
22	I	10.0	5.41	20.26	3.74	11.5 grams formic acid as Na salt given subcuta- neously in 50cc.
22	II		5.08	18.50	3.64	
23	III		5.27	17.29	3.28	
23	IV		5.05	16.72	3.31	

EXPERIMENT V. Twelve-hour periods.

Dec.	DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D:N	NH ₄ N	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	REMARKS
	14	I	13.3	5.62	25.68	4.57	0.21	0.109	0.686	10.0 grams normal butyric acid (Merck) as Na salt given subcutaneously in one injection.
	14	II		5.50	23.60	4.31	0.22	0.164	0.907	
	15	III	13.0	5.27	24.63	4.67	0.056	0.360	2.09	
	15	IV		5.34	21.35	4.00		0.162	1.16	11.5 grams formic acid as Na salt given subcutaneously.
	16	V	12.8	5.85	22.15	3.78		0.155	1.81	
	16	VI		5.42	20.27	3.74		0.098	2.53	

EXPERIMENT VI. Twelve-hour periods.

Dec.	DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D:N	NH ₄ N	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	REMARKS
	1	I	11.2	6.84	25.92	3.79	0.536	0.348		20.0 grams normal butyric acid as Na salt given subcutaneously in two doses.
	1	II		6.78	22.51	3.17		0.372	2.47	
	2	III		6.38	21.00	3.29	0.41	0.336	2.44	
	2	IV	9.5	6.18	20.82	3.37	0.18	0.599	3.30	
	3	V	Dog in deep coma. Exitus.							

EXPERIMENT VII. Twelve-hour periods.

Nov.	DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D:N	NH ₄ N	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	REMARKS
	2	I	13.07	5.68	19.23	3.38	0.17	0.295	1.38	9.2 grams normal valeric acid as Na salt given subcutaneously.
	3	II		4.78	24.13	6.06	0.158	0.227	1.50	
	3	III	12.2	4.67	18.54	3.97	0.32	0.324	1.71	

EXPERIMENT VIII. Twelve-hour periods.

Oct.

DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D.N	NH ₄ N	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY- BUTYRIC ACID	REMARKS
7	I	17.00	7.14	28.04	3.92	0.308	0.147	0.692	9.6 grams normal caproic acid as Na salt given subcutaneously.
7	II		7.12	27.30	3.84	0.33	0.275	1.24	
8	III	16.46	6.65	25.93	3.89	0.35	0.575	2.13	

Nov.

EXPERIMENT IX. Twelve-hour periods.

DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D.N	NH ₄ N	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY- BUTYRIC ACID	REMARKS
10	I	16.1	5.14	18.28	3.55	0.288	0.225	0.86	14.2 grams normal valerianic acid as Na salt dissolved in 50 cc. of water given subcutaneously in one dose.
10	II		4.96	26.20	3.28	0.139	0.100	1.41	
11	III		5.41	21.08	3.89	0.162	0.247	1.80	10.3 grams normal caproic acid as Na salt given subcutaneously.
11	IV	14.70	5.35	19.48	3.64	0.198	0.283	2.74	
12	V		4.85	18.62	3.88	0.160	0.684	3.13	13.0 grams normal heptylic acid as Na salt dissolved in 75 cc. of water.
12	VI	13.80	4.89	17.86	3.65	0.181	0.76	4.32	
13	VII		4.13	19.15	4.63	0.153	0.416	3.35	Lost
13	VIII	13.24	3.72	14.38	3.86	0.188	0.564		
14	IX		4.20	14.84	3.53	0.242	0.948	3.83	
14	X		3.90	14.79	3.79	0.220			

EXPERIMENT X. Twelve-hour periods.

DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	NH ₄ N	ACETONE, β -HYDROXY AND ACETO- ACETIC ACID	REMARKS
9	I	8.46	4.21	15.63	3.71	0.170	0.96	13.0 grams normal heptylic acid as Na salt given subcutaneously in two doses.
9	II		5.00	20.99	4.20	0.106	1.91	
10	III		4.26	16.44	3.86	0.202	3.13	
10	IV		4.43	16.94	3.82	0.200	3.58	
11	V		4.18	15.23	3.64		2.84	

STUDIES IN COTTON SEED MEAL INTOXICATION. I.¹

PYROPHOSPHORIC ACID.

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WITH THE COLLABORATION OF R. S. CURTIS AND G. A. ROBERTS.

(From the North Carolina Agricultural Experiment Station, Raleigh, N. C.)

(Received for publication, January 2, 1913.)

The injurious effect of the continuous feeding of cotton seed meal to calves and swine has been known for years. The cause has been ascribed by various investigators² to lint, oil, high protein content, a toxalbumin, choline, betaine, resin and decomposition products. Crawford³ in a preliminary paper published in March 1910 states that "The chief poisonous principle in certain cotton seed meals is a salt of pyrophosphoric acid." Crawford's conclusion is based upon the study of an extract obtained by digesting the meal at body temperature one day with pepsin and one day with pancreatin. He fed with a catheter extracts of cotton seed meal, these extracts corresponding to amounts of meal very much in excess of those which would be fed ordinarily. He made no study of the undissolved residues.

We began the study of the subject in 1908 and, since that time, have used many solvents in our efforts to extract the toxic substance. The undissolved residue having proved toxic in every case, we decided to investigate the residue undissolved by the pepsin-pancreatin treatment. We also tried other feeds which bear upon the question.

The animals selected were rabbits. Our normal daily feed for each animal was 15 grams of cotton seed meal or an amount of some fraction equivalent to 15 grams of meal. As the rabbits used averaged about 1.5 kilos the daily feed corresponds to 10

¹ This paper was read before the Biological Chemical Section of the American Chemical Society at the Washington meeting, December, 1911 (except the results of the last experiment on sodium pyrophosphate).

² *Exp. Sta. Record*, xxii, p. 502, 1910.

³ *Journ. of Pharmacol.*, i, p. 547, 1910.

grams per kilogram of initial live weight. The unconsumed feed was estimated and correction made. Each animal was allowed *per vires*, cabbage leaves or other green feed each morning. We mixed molasses with the cotton seed meal and, when some fraction of the meal was being fed, the portion of meal removed was replaced by an equivalent weight of wheat bran. Six controls were run during the entire time of the experiment and all the control animals lived. The feeds, daily and total, are all calculated to the equivalent in grams of cotton seed meal per kilo of initial weight of animal.

Pepsin and pancreatin extract.

Raw cotton seed meal was digested one day each with pepsin and pancreatin. The mass was filtered, and the filtrate concentrated to small volume. The solution was fed through a catheter in amounts corresponding to 200 grams of meal.

Nine rabbits were taken, varying in weight from 1461 to 2240 grams, average 1752. All lost in weight an average of 123 grams. Two were made very sick but survived, two showed no ill effect except loss in weight and five died. The amount fed corresponded to ten times our normal feed of cotton seed meal. The P_2O_5 in the feed was 2.22 grams.

Sodium pyrophosphate.

Sodium pyrophosphate was prepared in the laboratory by igniting disodium phosphate. The product responded to the tests for pyrophosphate and for freedom from orthophosphates. 4.157 grams corresponded to 2.22 grams of P_2O_5 , the amount contained in the pepsin-pancreatin extract of 200 grams of meal. The pyrophosphate for each animal was dissolved in 65 cc. of water and fed through a catheter.

One rabbit weighing 2070 grams died during the night following feeding. Another rabbit weighing 1456 grams, similarly fed, died thirty-one minutes afterwards.

The results of the preceding experiments indicate that the pepsin-pancreatin extract of 200 grams of meal is generally toxic when given at one feeding and that the $Na_4P_2O_7$ corresponding to the amount of P_2O_5 in that amount of meal is toxic if administered at a single feeding.

Cotton seed meal.

Twelve rabbits were taken whose initial weights ranged from 970 to 2560 grams, the average being 1559 grams. All the animals died within from 8 to 21 days, average, 13 days. The average loss in weight was 379 grams. The total amount of meal consumed by each animal ranged from 105 to 225 grams, average, 157 grams. The total amount eaten was practically 100 grams per kilo of initial weight of the animal, making an average daily consumption of meal equivalent to 7.7 grams per kilo of animal. These figures indicate the degree of toxicity of the meal towards the animals under the conditions of the experiment. There were 2.76 grams of P_2O_5 in the average feed or 0.21 gram in the daily feed.

Sodium pyrophosphate corresponding to whole meal.

Our cotton seed meal contained 1.76 per cent of P_2O_5 . If it were all in the form of pyrophosphate, 0.4157 gram of sodium pyrophosphate containing 0.222 gram of P_2O_5 would contain an amount equivalent to 12.5 grams of meal.

Four rabbits varying from 790 to 1550 grams, averaging 1117 grams, were fed daily 0.4157 gram of $Na_4P_2O_7$. Each animal gained in weight, the average gain being 243 grams. At the end of fifty-two days the feed was discontinued, all the animals being in good condition. For each kilo of animal the equivalent of the feed in cotton seed meal was a total of 582 grams; daily, 11.2 grams.

This feed furnished each animal daily with more pyrophosphoric acid than the amount received by each animal eating the raw meal, yet this feed was non-toxic and the meal toxic. This indicates that pyrophosphoric acid is not the cause of toxicity in cotton seed meal.

Our next step was to ascertain which was the more toxic portion of the meal, the aqueous extract, the pepsin-pancreatin extract of the residue undissolved by water or the residue undissolved after both of these treatments.

Aqueous extract of cotton seed meal.

1260 grams of cotton seed meal were stirred at room temperature with 1050 cc. of water and 10 cc. of chloroform for twenty-

four hours. The solution was filtered, the liquid evaporated over a water bath to a syrup, poured over 240 grams of bran, mixed thoroughly, dried and fed to each animal in amounts corresponding to 30 grams of cotton seed meal daily—twice our normal feed. Six animals were taken. One animal died after 16 days, having lost 369 grams. The death being from causes other than the feed the observations are not included. The other five animals ranged in weight from 1137 to 1947 grams, average, 1400. The feed was discontinued after thirty-eight days, the animals having gained an average of 203 grams. There were 2.26 grams of P_2O_5 in the total average feed or 0.06 gram in the daily feed.

Pepsin and pancreatin extract.

The residue left upon the filter in preparing the preceding feed was washed thoroughly with hot water and then digested at 40° for one day each with pepsin and pancreatin. The mass was filtered, the filtrate evaporated over a water bath to a syrup, poured over bran and dried. Each animal was fed daily the amount corresponding to 30 grams of cotton seed meal—double our normal.

Six rabbits were taken ranging in weight from 1516 to 1864 grams, average, 1683. At the end of thirty-eight days the feed was discontinued, the animals having gained an average of 73 grams each.

There were 5.22 grams of P_2O_5 in the total feed or 0.14 gram in the daily feed.

Residue undissolved by treatment with water, pepsin and pancreatin.

The mass left after filtering off the solution from which the preceding pepsin and pancreatin extract feed was prepared was washed with hot water and dried. It was then finely ground in a mill. 8.8 grams, corresponding to 15 grams of meal, were mixed with bran and molasses and fed to each of six rabbits. The rabbits ranged in weight from 1374 to 1773 grams, average, 1592 grams. Five died in 14 to 16 days and the remaining one died at the end of 27 days—the average of all being 18 days. The average loss in weight was 325 grams. The total feed consumed corresponded to cotton seed meal equivalent to 116 grams per kilo initial average weight of animal, the average for a day being 6.4

grams per kilo. The total P_2O_5 consumed by the average was 1.96 grams, the average for a day being 0.11 gram of P_2O_5 .

It is thus seen that the residue undissolved by the treatment of the meal with water, pepsin and pancreatin solutions is toxic, but both of the extracts, although they corresponded to almost three or four times as much meal, were non-toxic.

The total P_2O_5 in the toxic portion (1.96 grams) was less than in either of the non-toxic fractions.

Residue undissolved by pepsin and pancreatin.

Cotton seed meal was digested as in our first experiment with pepsin and pancreatin. The mass was filtered and the residue was washed with water, dried and ground. 10.7 grams corresponded to 15 grams of meal. Three rabbits were taken, weighing 1860 and 2289 grams, average, 2124 grams. All lost in weight, the average being 552 grams, and all died in an average of 21 days—the range being from 15 to 27 days. The total feed per kilo of animal corresponded to an equivalent of 128 grams of cotton seed meal, the average daily feed being 6.1 grams per kilo. There were in the total average feed 1.40 grams of P_2O_5 , making a daily average of 0.07 gram.

This feed resembled the preceding feed closely, both in composition and results, and confirms our conclusions that the most toxic part of the meal is in the residue undissolved by pepsin and pancreatin.

We next prepared a feed which had an inappreciable amount of P_2O_5 and yet which was toxic. Our solvent was ammonium citrate solution.

Residue after citrate extraction.

450 grams of cotton seed meal were extracted with 1500 cc. of a solution of ammonium citrate following the A. O. A. C. method for determining insoluble phosphoric acid. The mass was filtered, the residue washed and dried. 7.8 grams were the equivalent of 15 grams of meal.

Five rabbits were fed on this mixture. The weights ranged from 1350 to 2380 grams, average, 1602 grams. All lost in weight, the average loss being 438 grams. All died in from 17 to 29 days, the average being 21 days. The total feed equivalent in cotton

seed meal was 168 grams and the daily feed 8.0 grams per kilo of initial live weight.

The total P_2O_5 consumed by each was 0.60 gram, a daily average of 0.03 gram. This feed was almost as toxic as the whole meal or the pepsin-pancreatin residue, although it contained but an insignificant amount of P_2O_5 .

Sodium pyrophosphate.

The residue after ammonium citrate extraction contained a total of 0.60 gram of P_2O_5 in the amount consumed in twenty-one days. If all the P_2O_5 were in the form of pyrophosphate it would be the equivalent of 1.11 grams of $Na_4P_2O_7$. This amount dissolved in 60 cc. of distilled water was fed on January 17, 1912, at one feeding, through a catheter, to each of six rabbits, whose average weight was 1827 grams. The weights ranged from 1732 to 2037 grams. The average gain for each animal was three grams. The animals did not seem to experience any ill effects from the feed.

As the citrate residue, which proved toxic in 21 days, contained a total of 0.60 gram of P_2O_5 in the form of $Na_4P_2O_7$, and as this amount of P_2O_5 given at a single feeding to each of six rabbits proved harmless, the cause of the toxicity of the citrate residue is not pyrophosphoric acid.

SUMMARY.

Experiments show that the pepsin-pancreatin extract of cotton seed meal is toxic generally to rabbits, when fed in amounts corresponding to from fifteen to twenty times the amount of meal normally fed, and that the pyrophosphoric acid corresponding to this extract is toxic if fed similarly.

The pepsin-pancreatin residue is more toxic than the aqueous or pepsin-pancreatin extracts of the meal—in fact it is the only one of the three fractions which is toxic under the conditions of the feeding.

A fraction of cotton seed meal containing a non-toxic amount of pyrophosphoric acid may be toxic.

The results indicate that pyrophosphoric acid is not the cause of toxicity of cotton seed meal.

THE INFLUENCE OF FUNCTION ON THE LIME REQUIREMENTS OF ANIMALS.¹

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(Received for publication, January 4, 1913.)

A question which has baffled pathologists for a long time, and one which is not yet in a fair way of solution, is the cause of specific bone diseases known as rachitis, osteoporosis and osteomalacia, diseases which are all characterized by an impoverishment of the bones in inorganic substances. It is not desired to go into a discussion of the etiology of these pathological conditions, as a review of the literature will be found in various treatises,² but it is desired to correlate observations made in the study of these diseases with some experiments conducted with domestic animals designed to establish their minimum requirement of lime.

In regard to the amount of lime needed by an organism to enable it to maintain itself in lime equilibrium, we find no unanimity of opinion. It is evident that the lime requirements as well as the requirements for other inorganic substances vary with the physiological condition and activities of the animal. *A growing animal, a starving animal, a pregnant animal and a milk-producing animal, each has its specific drains upon the mineral nutrients.*

Undoubtedly an immature and growing animal demands a greater supply of lime in its ration than a mature one. Indeed the demand for lime in a dog has been found to stand in a direct relation to the rapidity of its growth.³ All investigators agree that with a growing animal a rachitic condition of the bones can

¹ Published by permission of the Director of the Agricultural Experiment Station.

² McCrudden: *Archives of Internal Medicine*, v, p. 596, 1910; Voit: *Zeitschr. f. Biol.*, xvi, p. 55, 1880; Aron: *Biochem. Zeitschr.*, xii, p. 28, 1908; Dibbelt: *Arbeit. a. d. path. Inst. Tübingen*, vii, p. 144, 1909.

³ Aron and Sebaauer: *Biochem. Zeitschr.*, viii, p. 1, 1908; Voit: *Zeitschr. f. Biol.*, xvi, p. 55, 1880.

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be very readily obtained by feeding a low lime ration.⁴ With mature animals there is no such uniformity of opinion. Dibbelt,⁵ working with a mature dog weighing 28 pounds, secured a positive balance of 0.1 gram of CaO over a ten-day period with a meat-lard diet supplying but 0.05 gram of CaO per day. Heiss⁶ kept a mature dog weighing 8.8 pounds for a period of 308 days practically in lime equilibrium on a ration supplying 0.042 gram of CaO per day. Forster,⁷ Voit,⁸ Müller⁹ and Kochmann,¹⁰ on the basis of their experimental data, maintain that such a low lime ration cannot satisfy the requirements of even a mature animal and present protocols showing negative balances on even high lime rations. So far as we are aware, however, there are no tissue analyses at hand showing that the skeleton of a mature animal can be thus impoverished.

With starving animals there is a continual loss of lime in the urine as well as in the feces. Munk,¹¹ working with a dog, observed a loss of 0.176 gram in the feces and 0.029–0.096 gram in the urine daily for a ten-day period. With the professional faster, Cetti, he¹² observed a loss of 4.08 grams of CaO in ten days and with Breithaupt, 0.561 gram in six days.

With pregnant animals it is well known that a developing fetus constitutes an intense drain upon the lime supply of the mother, which may be sufficiently impoverished in this manner to show intense pathological symptoms.¹³

THE INTESTINAL FACTOR IN DETERMINING THE MINIMUM LIME REQUIREMENT OF ANIMALS FOR MAINTENANCE.

Data collected from all sources show, without exception, that our domestic animals require a continual renewal of the supply of inorganic elements to repair the losses incurred in metabolism.

⁴ Voit, Aron, McCrudden, Dibbelt: *loc. cit.*

⁵ Dibbelt: *loc. cit.*

⁶ Heiss: *Zeitschr. f. Biol.*, xii, p. 165, 1876.

⁷ Forster: *Zeitschr. f. Biol.*, ix, p. 297, 1873.

⁸ Voit: *loc. cit.*

⁹ Müller: *Zeitschr. f. Biol.*, xx, p. 327, 1884.

¹⁰ Kochmann: *Biochem. Zeitschr.*, xxxii, p. 10, 1911.

¹¹ Munk: *Pflüger's Archiv*, lviii, p. 309, 1894.

¹² Munk: *Virchow's Archiv*, cxxxi, Supplement, p. 168, 1893.

¹³ McCrudden: *loc. cit.*

In all bone diseases where the animal may suffer extreme agony for want of lime it has been observed that during starvation and during periods of low lime feeding there is a continual lime loss, infinitesimal at times in the urine but always considerable in the feces. Even in prenatal life, lime accumulates in the meconium to a very considerable extent.¹⁴ Why, in all these cases, the lime is not reabsorbed and re-utilized by the cells which demand it, is problematical as, theoretically, there is evident no chemical reason why the lime should not be available. It cannot be merely a question of solubility and that certain insoluble and therefore unavailable compounds are formed; in a salt solution, such as the body fluids, there is a continual redistribution in the combinations between acid and base radicals with the removal of any one component. It seems far more plausible to look upon this excretion of (unavailable?) salts as imposed on the animal by physical and physiological processes. Every cell, in the selection of its nutrients from a solution, has to perform a certain amount of work to overcome purely physical factors of solution and osmosis. Before this selection to the extent of the exhaustion of the desired element has taken place, the source of supply may have been removed and an excretion is observed under conditions of actual want. Specifically, the authors are inclined to concur with Dibbelt¹⁵ in his idea that the lime excreted in the feces even during rachitis has as its source normal secretions of the intestinal tract. Due to abnormalities in absorptive processes this lime is not reabsorbed to the usual extent and consequently makes its appearance in the feces. In support of this point of view we have the numerous observations that rachitis is usually preceded by visible digestive disturbances. Voit¹⁶ evidently was of a similar opinion as he said, "It is evident that the body is offered too small an amount of lime, not only in the case of an insufficiency of lime in the ration, but also when too small an amount of the ingested lime is absorbed through any cause as, for example, digestive disturbances or diarrhoea or defective assimilation from excessive feces-yielding nutrients." How digestive disturbances in themselves can call forth the excretion of lime into the intestine is not evident.

¹⁴ Müller: *Zeitschr. f. Biol.*, xx, p. 327, 1887.

¹⁵ Dibbelt: *Habilitationsschrift*, Tübingen, 1903.

¹⁶ Voit: *Zeitschr. f. Biol.*, xvi, p. 117, 1880.

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It seems far more probable that it merely inhibits absorptive processes. That, under certain conditions, excessive secretion of lime into the intestine cannot take place is not maintained as it is known that there is an actual secretion or excretion by this channel of numerous substances introduced into the animal body.¹⁷ The idea that the intestine may in certain respects function as a kidney is not a new one,¹⁸ but substances, foreign or native to the body, introduced in excessive amounts are not to be compared in their method of elimination with normally occurring processes. Indeed, Mendel and Benedict¹⁹ found that the injection of calcium salts was not always accompanied by increased calcium in the feces.

With domestic farm animals feeding on plant materials, it can readily be seen how the bulky digestive residues originating from these may hinder efficient absorption of secreted, lime-containing digestive juices. In this manner a feeding stuff high in indigestible material may require a higher initial lime content to enable it to maintain a positive lime balance for the animal than one composed of largely digestible materials. Again, the investigator is handicapped by lack of information on the form in which our inorganic materials are found in plant tissues. With oat straw,²⁰ all the lime has been found soluble in $\frac{N}{16}$ HCl, which gives us an idea of its solubility in the gastric secretion. Of its absorbability we cannot venture any statements. There are too many unknown factors which may influence the absorption of lime, of which the rôle of the various forms of *silica* in plant tissues may not be the least important.

Experimental.

In view of the fact that no metabolism experiments have ever been conducted to determine the minimum lime requirements of farm animals as determined by intestinal excretion, it was considered desirable to secure such data with the pig and goat. The pig, above all animals, is most liable to be subject to an insufficiency of lime in the ration.

¹⁷ Mendel: *Amer. Journ. of Physiol.*, xi, p. 5, 1904.

¹⁸ Liebig: *Chem. Briefe*, 1845, pp. 303-4.

¹⁹ Mendel and Benedict: *Amer. Journ. of Physiol.*, xxv, p. 23, 1909.

²⁰ Unpublished data.

EXPERIMENT I. To secure these data a 75-pound pig was fed a ration initially very low in lime, but to which calcium phosphate was added in successively increasing amounts. Records of lime excretion were kept quantitatively for seven-day periods on a maximum consumption of the ration.

Composition of ration: 1.8 pounds of rice, 0.2 pound of corn meal, 0.12 pound of wheat gluten.

The pig had previously been fed on the standard University ration, consisting of 30 parts corn, 30 parts oats, 30 parts middlings and 10 parts oil meal. On November 6 the ration was changed. Owing to the digestible character of the feed but little feces were excreted for a few days, which was mistaken for constipation. To remedy this 12 grams of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were added to the feed on November 12, and 15 grams, on November 13. On November 18 the first daily collection was made.

Though the intake of lime in the low-lime period was as low as was possible to secure, when supplying the necessary energy and nitrogen intake on unextracted plant materials, there was always observed a positive balance. In period V a negative balance appears to have prevailed, due merely to the carrying over in the gut of unexcreted lime from the preceding period. As has

TABLE I.
Record of lime balance. Daily averages in grams.

	CAO IN FEED AND WATER	CAO IN FECES	CAO IN URINE	CAO RETAINED
I. Low lime ration. No additions. Nov. 18-Nov. 24....	0.245	0.208	0.012	+0.025
II. Low lime ration + 2 grams $\text{Ca}_3(\text{PO}_4)_2$ Nov. 25-Dec. 1...	0.897	0.207	0.016	+0.674
III. Low lime ration + 4 grams $\text{Ca}_3(\text{PO}_4)_2$ Dec. 2-Dec. 8....	1.673	0.347	0.013	+1.313
IV. Low lime ration + 8 grams $\text{Ca}_3(\text{PO}_4)_2$. Distilled water. Dec. 9-Dec. 15.....	2.916	0.688	0.023	+2.205
V. Low lime ration. Distilled water. No additions. Dec. 16-Dec. 20.....	0.203	0.708	0.013	-0.518
VI. Low lime ration. Distilled water. No additions. Dec. 21-Dec. 29.....	0.203	0.091	0.010	+0.102

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been observed before,²¹ calcium phosphate offers a readily available source of lime to the pig. Here is brought out the additional fact of the tenacity with which lime once stored is retained, as no increased excretion was observed in the succeeding low-lime period. In fact, the lime content of the feces was even lower in the last low-lime period than in the initial. The reason for this is not evident unless the administration of the cathartic in the first period was an important factor. No excessive purging was observed.

EXPERIMENT II. In experiment I was seen the failure of a low lime ration to cause a negative lime balance. In experiment II an attempt was made to reduce the lime intake still more by the use of properly acid-extracted plant materials of an initial low lime content. Commercial starches were found to average higher in lime than ordinary rice, which by extraction with dilute HCl and then repeated washing with distilled water to neutrality was lowered in lime content from 0.0103 per cent to 0.0049 per cent. Protein was supplied in the form of similarly extracted wheat gluten which was reduced to a lime content of 0.015 per cent. To prevent those physiological disturbances liable to occur on feeding a ration practically salt-free, a salt mixture containing 3 grams of K_2HPO_4 , 3 grams of NaCl and 1 gram of $MgSO_4 \cdot 7H_2O$ was added to the daily ration.

Pig I. Growing pig. Initial weight, 111 pounds; final weight, 112 pounds. The composition of the daily ration was: 943 grams of extracted rice, 0.0049 per cent CaO; 56 grams of extracted gluten, 0.015 per cent CaO; 7 grams of salt mixture.

TABLE II.

Lime balance. Daily averages in grams. (Low lime ration fed, beginning February 9.)

	CaO IN FEED	CaO IN FECES	CaO IN URINE	CaO RETAINED
February 17-23.....	0.054	0.097	0.030	-0.073

Pig II. Mature pig. Initial weight, 312 pounds; final weight, 318 pounds. The composition of the daily ration was: 2452 grams of unextracted rice, 0.0212 per cent CaO; 272 grams of extracted gluten, 0.0282 per cent CaO; 18.2 grams of salt mixture.

²¹ Hart, McCollum and Fuller: Research Bulletin 1, Wisconsin Experiment Station.

TABLE III.

Lime balance. Daily averages in grams. (Low lime ration fed, beginning December 2.)

	CaO IN FEED	CaO IN FECES	CaO IN URINE	CaO RETAINED
December 11-24.....	0.597	0.666	0.052	-0.121

Here in two specific cases the level of lime intake was brought below the amount necessary to obtain lime equilibrium. Of the specific forces which brought about these losses, both through the kidney and through the gut, nothing can be said at present. Whatever they are, they are as persistent in the pig as in other animals, which becomes evident when the intake is sufficiently reduced. From the data secured, a daily intake of 0.3 gram of CaO per 100 pounds body weight would cover these losses under the above conditions. What the requirements would be for more indigestible rations or for rations with a different accompanying salt content cannot be definitely stated.

THE FACTOR OF MILK PRODUCTION IN DETERMINING THE MINIMUM
LIME REQUIREMENTS OF AN ANIMAL FOR ITS MAINTENANCE.

In considering the influence of function upon lime metabolism not the least interesting and important is the factor of milk production. In a previous publication,²² as well as in subsequent verifications of these data,²³ it has been shown that with milk-producing animals, on certain rations, a very pronounced negative lime balance may be prevailing over extended periods of time. This, in its intensity, may assume much importance and necessitates further consideration of the principles underlying lime metabolism. In this problem consideration should be given to the relative efficiency of absorption of the mineral elements, the variations in the occurrence in the urine of various acids and bases and the interrelations prevailing among elements in their physiological capacities during and after lactation. All of these are of fundamental significance. It is hoped in some measure to throw further light on these relations in this article by

²² Hart, McCollum and Humphrey: Wisconsin Experiment Station, Research Bulletin 5, 1909.

²³ Fingerling: *Landw. Versuchsstat.*, lxxv, p. 1, 1911.

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a reconsideration of previous data as well as by the presentation of new observations.

In regard to the interrelations prevailing among mineral elements in metabolism during lactation, data from this station, as well as those published by Rose,²⁴ give us some very interesting material. On a low phosphorus intake, with a heavy milk production, there invariably was observed the simultaneous occurrence of a large increase in the lime excreted in the urine over that of a previous period with a high phosphorus intake. A high urinary excretion of lime under conditions of actual want has never been previously observed except under conditions of prevailing acidosis where naturally an excessive demand is made upon the bases to maintain the neutrality of the tissues.

Acidosis as the cause of this high lime excretion is out of the question as the urine was always found distinctly alkaline in reaction. Here the high output of lime in the urine is to be considered as the direct result of an insufficient phosphorus assimilation at a time when the demand for this substance was augmented by milk secretion. As often observed in other connections, in an attempt to maintain the secretion of the mammary gland normal, the demands of the gland take precedence over the nutritive requirements of the body. Here, as a specific case in mineral metabolism, we have the mammary gland removing phosphorus from the body tissues in an endeavor to maintain its secretion normal in amount as well as in composition. This much our data show. As to the source of the phosphorus withdrawn we can merely speculate. *A priori*, we are led to believe that it was derived from skeletal tissues. In bone there is 1.72 times as much lime as in milk, in proportion to their phosphorus pentoxide content. Naturally, then, with the utilization of phosphorus from the skeleton for milk production, an excess of lime would be liberated and recovered in the excreta, which is exactly what was observed. Apparently all the excess of lime liberated made its appearance in the urine. If under any conditions the walls of the intestine function as an excretory organ for lime it is not evident in this connection. With the urine and also very probably the blood heavily laden with lime, we would certainly have reason

²⁴ Rose: N. Y. (Geneva) Exp. Station, Technical Bulletin 20, 1912.

to expect an increased excretion with the feces; but the amounts of lime in the feces do not show any consistent relation to the increased lime output in the urine. In summarizing the principles enunciated it may be briefly said that an inorganic substance such as lime may be excreted in increased amounts when in the excessive metabolism of another element it becomes a real waste product of metabolism.

To accumulate more data on the absorption and excretion of lime on different rations and with the animal serving different functions, a new series of experiments was carried out using the goat as the experimental animal. This made it possible to make all collections of excreta with the metabolism cage previously used in studying the lime nutrition of the pig.

Plan of the experiment. The goat was confined in the metabolism cage throughout the experimental period in connection with a study of creatinine metabolism during and after lactation. This necessitated the inclusion of some periods not immediately germane to the subject under consideration. The different rations enumerated were fed in succession and quantitative collections of the excreta made daily. Analyses of the excreta for lime were made in each period, when possible, only after a suitable lapse of time—usually five days—since feeding the preceding ration. Nitrogen determinations on the milk as well as on the excreta were made daily beginning April 17 and continued throughout the experiment.

A goat in full milk flow, producing about two quarts of milk daily on a corn, oat and clover ration, was selected as a suitable animal. Her quiet and tractable nature made her especially able to endure the long confinement in the metabolism cage, with little inconvenience and that shown only toward the close of the period. But in spite of the prevailing opinion that a goat has no epicurean tendencies it was found impossible to obtain a consumption of the ration of bran, rice, wheat gluten and oat straw on which the cow, previously referred to, had been fed. Though for various reasons it was highly desirable to duplicate the ration as nearly as possible, a considerable reduction in the amount of bran and the addition of some corn was found necessary to secure consumption.

The periods involve two phases of experimentation. The first two are records of the animal in full milk. In period III the milk

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flow was gradually being reduced. The periods following III are records with complete or almost complete cessation of mammary function.

Rations fed.

PERIOD I. *April 4-May 10.*

7.5 pounds of wheat bran	} 0.030 per cent CaO.
45.0 pounds of corn meal		
30.0 pounds of rice meal		
17.5 pounds of gluten meal		
25.0 pounds of oat straw.....		0.472 per cent CaO.

An approximate average of 1.6 pounds of grain and 0.4 pound of straw consumed daily.

PERIOD II. *May 11-May 17.*

Same ration as in I with straw intake doubled.

An approximate average of 1.6 pounds of grain and 0.8 pound of straw consumed daily.

PERIOD III. *May 18 - May 31.*

8.0 pounds of corn meal	} 0.028 per cent CaO.
5.0 pounds of rice meal		
1.0 pound of wheat bran		
1.0 pound of gluten meal		
10.0 pounds of oat straw.....		0.472 per cent CaO

An approximate average of 1.2 pounds of grain and 0.8 pound of straw consumed daily.

PERIOD IV. *June 1-June 11.*

6.0 pounds of corn meal;
6.0 pounds of rice meal;
8.0 pounds of oat straw.

An approximate average of 1.2 pounds of grain and 0.8 pound of oat straw consumed daily.

PERIOD V. *June 12-July 2.*

Straw only.

An approximate average of 1.2 pounds of straw consumed daily.

PERIOD VI. *July 3-July 29.*

Same ration as in Period I.

An approximate average of 0.8 pound of grain and 0.2 pound of straw consumed daily.

PERIOD VII. *July 30-August 17.*

Allowed to graze during the day and fed in addition 1.2 pounds of oats daily.

PERIOD VIII. *August 18-September 5.*

Same ration as in Period I and VI.

An approximate average of 0.8 pound of grain and 0.2 pound of straw consumed daily.

TABLE IV.

Record of lime balance in the several periods and daily averages.

	CaO IN FEED	CaO IN WATER	CaO TOTAL INTAKE	CaO IN EX- CRETA	CaO ABSORBED	CaO IN MILK	CaO IN EX- CRETA* AND MILK	CaO BALANCE
	grams	grams	grams	grams	grams	grams	grams	grams
I. April 11- May 10....	1.07	0.18	1.25	1.10	+0.15	2.97	4.07	-2.82
II. May 11-17	1.92	0.15	2.07	1.48	+0.59	2.74	4.22	-2.15
III. May 18- 23.....	1.86	0.12	1.98	1.45	+0.53	1.71	3.16	-1.10
V. June 17-23.	2.56	0.10	2.66	2.52	+0.14	0.13	2.65	+0.01
VI. July 7-29..	0.55	0.06	0.61	1.54	-0.93	0.03	1.57	-0.96
VIII. August 24-Sept. 5....	0.60	0.06	0.66	0.42	+0.24	0.00	0.42	+0.24

* Lime in excreta, as tabulated, does not include the urinary excretion. This varied from traces to a maximum of 0.04 gram in very exceptional cases. Data on this were accumulated only for a total of about seven days in each period and were judged insignificant in the above connection. No tendencies to vary the urinary excretion in either direction by any of the rations were apparent.

TABLE V.

Nitrogen balances.

	N IN FEED	N OUTGO TOTAL	N BALANCE
	grams	grams	grams
I. April 20-April 26.....	126.07	127.36	- 1.29
April 27-May 3.....	135.71	131.68	+ 4.03
May 4-May 10.....	135.71	130.66	+ 4.05
II. May 11-May 17.....	142.50	133.55	+ 8.95
III. May 18-May 24.....	86.15	93.96	- 7.81
V. June 15-June 21.....	19.38	36.64	-17.27
June 22-June 23.....	20.35	28.55	- 8.29
VI. July 6-July 12.....	77.55	68.65	+ 8.90
July 13-July 19.....	69.13	61.69	+ 7.44
July 20-July 26.....	67.85	65.65	+ 2.20

Discussion. Period I. We have here a verification of results obtained with the cow: namely, milk production on a low lime ration results in a pronounced negative lime balance. Here also we have again the appearance of a large amount of fecal lime, the origin of which is not evident. A slight excess of lime intake over that excreted is observed, which for the sake of convenience is designated "lime absorbed." The table on nitrogen balance shows an actual retention in spite of the large milk secretion. The

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absorption of nitrogen and lime as indicated by the fecal excretion do not parallel. Apparently they are separate and distinct functions.

Period II. Essentially this is a continuation in results of the data in period I. The increased amount of straw in the ration, in spite of the assumed tendency for it to depress the reabsorption of lime containing secretions by the increase in fecal bulk, has actually increased the absorption of lime. Other factors must have come into play. These data are somewhat invalidated by the briefness of the period of observation, with the omission of the usual interval between periods. The fecal lime, however, was relatively constant in amount throughout this period, which would not have obtained if such factors had influenced the character of the data. It seems safe to conclude that the lime from oat straw was available to the goat.

Period III. This period marks the beginning of a reduction in the amount of protein fed, in an attempt to reduce the milk production for the later periods of observation. The utilization of lime and its balance does not show any new characteristics.

Period IV. Here we have a still further reduction in the protein ingestion, but as no analyses for lime were made this period need not occupy our attention.

Period V. To get a clearer insight into the availability of lime from straw this period ought to offer very satisfactory data, as the ration consisted exclusively of oat straw. We observe, however, but a very small utilization of the lime. The animal is only saved from a continuation of the impoverishment of its tissues in lime by the reduction in the milk flow. Upon comparison of the lime retention with the N retention one would say that here is to be found the explanation for the small positive lime balance observed. With a pronounced deficit in nitrogen, bone tissue could not be built up, consequently there was not any immediate demand for lime, in spite of the previous depletion.

Period VI. In continuation of this line of reasoning one would, in view of the generous N retention, expect a corresponding lime retention. The animal had been depleted in lime as well as nitrogen and now with the first opportunity a restoration of these elements would be expected. For nitrogen, this occurred, though the animal, already weighing scarcely 68 pounds, 11 ounces, con-

tinued to lose in weight until at the close of this period she weighed but 63 pounds. For lime there was a steady and consistent negative balance day after day with no indications of a change. The urinary lime excretion was not increased. Physical symptoms of osteomalacia were not observed although the animal showed a slight stiffness in the hind quarters, which was not surprising after such close confinement in a metabolism cage for a period of three months.

The feces were hard and dry and small in bulk; a slight failing in the appetite as well as a slight unresponsiveness to call or change in its environment began to be apparent. No definite results could be expected by longer continued observation under these conditions.

Period VII. On a ration of June grass and oats recovery speedily followed. In the course of two weeks with a gain in weight of 4 pounds all symptoms indicative of approaching collapse were dispelled. On the supposition that the change in physiological condition might influence her lime metabolism additional data were collected.

Period VIII. In view of the fact that no reliable results on the retention of nitrogen can be obtained over a short isolated period, no record of the nitrogen balance was kept. All indications were that the animal was in a good nutritive condition. Her eyes were bright, her senses acute and she showed a uniform gain in weight of 2 pounds during the period. Regular consumption of the ration was secured, and the feces were again of a normal consistency; as the table shows, a retention of lime now took place as a direct result of the accompanying change in the condition of the animal. This transformation is truly remarkable and from the standpoint of mineral metabolism stands unparalleled in the experimental field. From a nutritive condition which would ultimately have led to the occurrence of physical symptoms of osteomalacia, there was brought about a complete restoration to the normal by a temporary change in the ration. That the change of intake of lime was responsible for this transformation is out of the question, as the former ration, for maintenance, left little to be desired from the inorganic standpoint. Some subtle change in the trend of metabolism was instituted by the temporary variation in feed intake, as well as environment, which now enabled

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the animal to assimilate lime, formerly unavailable. From this point of view osteomalacia is not to be considered primarily a physiological disturbance brought about by the unavailability of lime in the source of supply, but rather the result of inefficient absorption of lime from the gut due to a complication of numerous factors. What these are we cannot venture to say. There was not a cessation of all absorptive processes in the intestine since in period VI we noted a generous utilization of the nitrogen from the ration in spite of the excessive loss of lime. An independence of specific assimilative functions could not possibly be more positive than in this connection. It is either a peculiar coincidence or else an important observation that with a pig in an experiment previously reported²⁵ a slight negative lime balance was likewise observed simultaneous with the excretion of hard and dry feces.

CONCLUSIONS.

1. The level of lime intake necessary for maintenance is dependent upon the functional activity of various organs of the body. A daily intake of about 0.3 gram of CaO per 100 pounds body weight covered the metabolism losses of a mature barren pig. From 0.4 to 0.5 gram of CaO per 100 pounds body weight covered the metabolism loss of a mature dry goat. The figures are not absolute and general, but will vary with the character of the ration.

2. The mammary gland during its activity constitutes a severe drain upon the skeletal lime supply during periods of insufficient lime assimilation. During periods of insufficient phosphorus assimilation, it indirectly causes a waste of lime from the skeleton.

3. An allowance of 1 gram of lime in the ration per pound of milk produced by a goat or cow should theoretically be ample. This, of course, is in addition to the maintenance requirement. But at least twice the above amount would be safer, due to the large losses of lime in the intestine accompanying increased food consumption.

4. The walls of the intestine with their normal secretions may cause the loss of a sufficient amount of lime to appreciably lower its "coefficient of digestibility" during periods of sufficient lime

²⁵ Research Bulletin 30, Wisconsin Experiment Station.

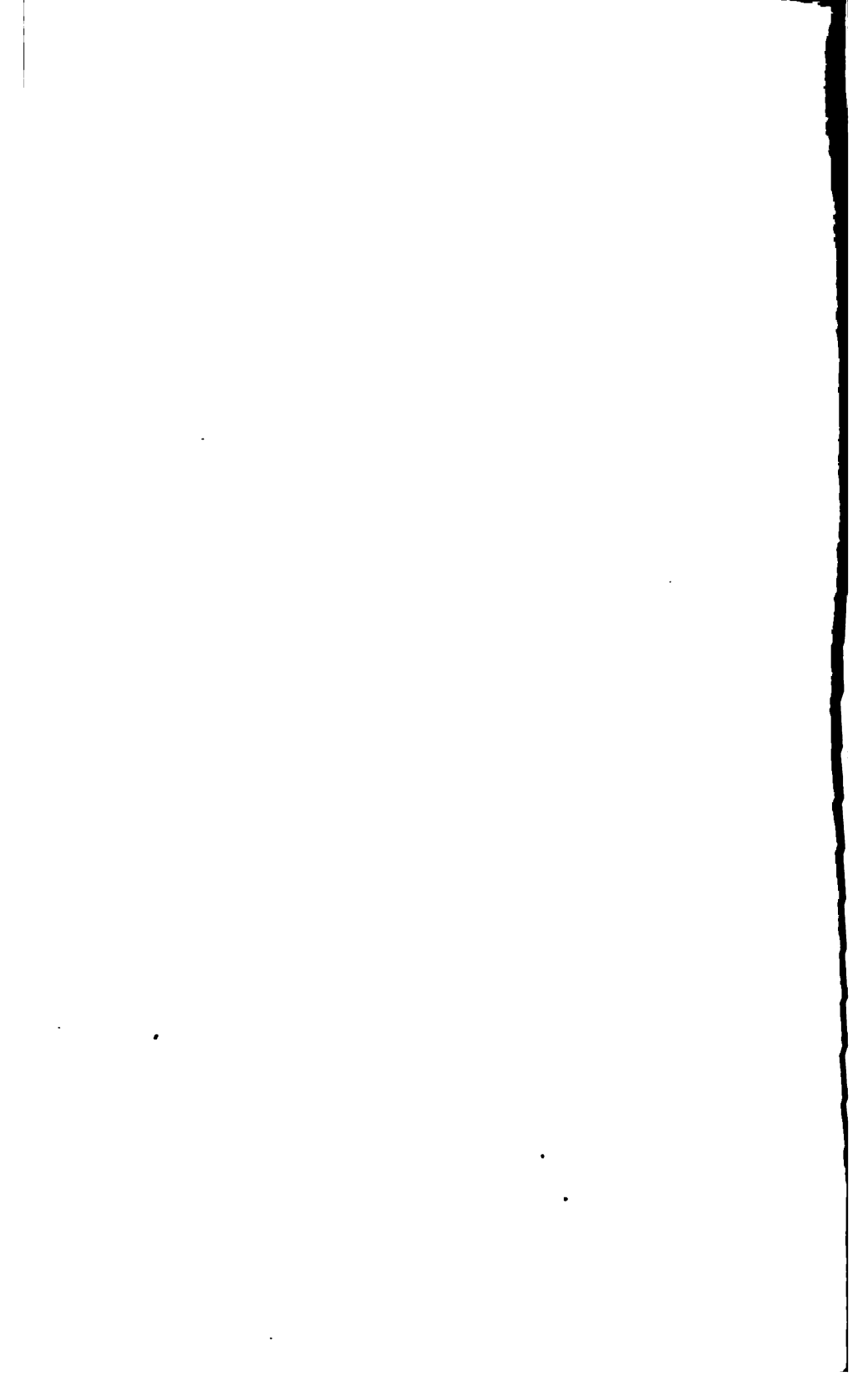
ingestion. When such conditions are complicated by physiological disturbances a large negative balance of lime over an extended period of time may result.

5. Under normal conditions with a low lime ingestion the usual intestinal losses may in themselves be the cause of a negative lime balance.

6. Intestinal and urinary losses of lime do not parallel. With very heavy intestinal losses the urinary excretion may remain unchanged.

7. A liberal assimilation of nitrogen does not necessarily imply an assimilation of lime even when the animal's supply of lime is considerably depleted. These are separate and distinct functions of the alimentary tract.

8. A perverted lime metabolism which ultimately would end in an extreme impoverishment of the skeleton in lime may be merely the result of other physiological disturbances.



THE EFFECT OF A HIGH MAGNESIUM INTAKE ON CALCIUM RETENTION BY SWINE.¹

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Wisconsin.)

(Received for publication, January 4, 1913.)

The experiment to be described in this paper relates to the influence which the addition of magnesium salts and of magnesium salts plus phosphates to the diet may exert upon calcium metabolism. They may also be interpreted as throwing some light upon the general problem of the mutual relationships which exist among the mineral constituents of the diet, considered from the standpoint of animal nutrition.

Many experiments are on record which indicate the existence of important biological relationships between calcium and magnesium. For example, Loew² showed that magnesium could serve in the nutrition of plants only in the presence of calcium salts. Loeb³ found the contractile reaction in jellyfish induced by magnesium to be inhibited by calcium. Meltzer and Auer⁴ proved that the effects of calcium on muscle and nerve in rabbits could be reversed by the injection of magnesium salts. In dogs, Mendel and Benedict⁵ observed that the injection of calcium salts was followed by increased elimination of magnesium in the urine: similarly injection of a magnesium salt increased the urinary output of calcium. Malcolm⁶ published evidence tending to show

¹ Published by permission of the Director of the Agricultural Experiment Station.

² Loew: U. S. Dept. of Agric., Div. Veg. Path. and Physiol., Bulletin 18.

³ Loeb: this *Journal*, i, p. 427, 1905-06.

⁴ Meltzer and Auer: *Amer. Journ. of Physiol.*, xxi, p. 400, 1908.

⁵ Mendel and Benedict: *Amer. Journ. of Physiol.*, xxv, p. 1, 1909.

⁶ Malcolm: *Journ. of Physiol.*, xxxii, p. 183, 1905.

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that ingestion of magnesium salts may cause a loss of calcium from adult animals and may hinder its deposition in the young. Forbes,⁷ basing his considerations upon these data, is inclined to believe that the cause of "bran disease," "shorts disease" and "millers horse rickets" is to be found in an excessive proportion of magnesium to calcium in bran and shorts.

Similarly, the influence of phosphates in the diet upon calcium metabolism is indicated in the work of Ingle⁸ who attributed the cause of a bone disease, manifested chiefly by impoverishment of the skeleton in ash, to a disproportionate content of lime and phosphorus pentoxide in the feeding stuff. Dibbelt⁹ observed the loss of 12.7 grams of lime in the feces alone from the body of a small dog when 5 grams of Na_2HPO_4 were added daily to its meat and fat diet. Bertram,¹⁰ working with a goat, likewise observed loss of lime from the body when Na_2HPO_4 was added to its ration.

In order to obtain data bearing upon Forbes' hypothesis mentioned above as well as to gain further information concerning the influence of variation in the mineral content of the ration upon calcium excretion, the following experiment was carried out.

The subject of the experiment was a pig, weighing at the beginning of the experiment 166 pounds, at the end, 156 pounds. Quantitative collection of excreta was made possible by confinement in a metabolism cage previously described by McCollum and Steenbock.¹¹ The bran ration fed throughout the experiment consisted of corn, 1.3 pounds; wheat bran, 2.4 pounds; oatmeal, 0.3 pound; and contained 0.134 per cent CaO ; 0.820 per cent MgO ; 2.924 per cent P_2O_5 . Of this ration 1 pound 11 ounces was consumed daily.

Although the proportion of MgO to CaO in the ration was high, it seemed advisable to accentuate the probable effect of the

⁷ Forbes: Ohio Experiment Station Bulletin 213, 1909.

⁸ Ingle: *Journ. of Agric. Science*, iii, 1908; *Journ. of Comp. Path. and Ther.*, xxi, xxii, 1907-08.

⁹ Dibbelt: *Arbeit a. d. Geb. d. path. anat. Inst. Tübingen*, vii, p. 559, 1911.

¹⁰ Bertram: *Zeitschr. f. Biol.*, xiv, p. 340, 1878.

¹¹ McCollum and Steenbock: Research Bulletin 21, Wisconsin Experiment Station.

magnesium in one part of the experiment by adding magnesium as the chloride; in another, magnesium was added as the sulphate. Later, in view of the depressing influence which phosphoric acid exerts upon the solubility of calcium and magnesium salts, a period was included in which the bran ration was supplemented by the addition of both magnesium salts and a soluble phosphate. Potassium phosphate was used in amount sufficient to combine with all the magnesium salts added to form tertiary magnesium phosphates.

RECORD OF CALCIUM EXCRETION.

DATE	CaO IN RATION	CaO IN FECES	CaO IN URINE	CaO IN EXCRETA	CaO RETAINED
	grams	grams	grams	grams	grams
<i>January. Bran ration only.</i>					
14	1.67	1.64	0.010	1.650	
15	1.67	1.47	0.059	1.529	
16	1.67	1.49	0.000	1.490	
17	1.67	1.37	0.000	1.370	
18	1.67	1.40	0.030	1.430	
<hr/>					
	8.35	7.37	0.099	7.469	+0.881
Daily average.....	1.67	1.47	0.019	1.490	+0.170

January. Bran ration + 6.08 grams of MgO as MgCl₂.

19	1.67	1.38	0.155	1.535	
20	1.67	1.41	0.276	1.686	
21	1.67	1.42	0.221	1.641	
22	1.67	1.56	0.269	1.829	
23	1.67	1.39	0.278	1.668	
24	1.67	1.24	0.262	1.502	
25	1.67	1.49	0.229	1.719	
26	1.67	1.69	0.258	1.948	
27	1.67	1.44	0.305	1.745	
28	1.67	1.51	0.342	1.852	
29	1.67	1.20	0.292	1.492	
30	1.67	1.70	0.253	1.953	
31	1.67	1.13	0.067	1.197	
<hr/>					
	21.71	18.56	3.207	21.767	-0.057
Daily average.....	1.67	1.42	0.246	1.673	-0.003

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RECORD OF CALCIUM EXCRETION—Continued.

DATE	CaO IN RATION	CaO IN FECES	CaO IN URINE	CaO IN EXCRETA	CaO RETAINED
	grams	grams	grams	grams	grams
February. <i>Bran ration only.</i>					
1	1.67	1.21	0.188	1.398	
2	1.67	1.75	0.091	1.841	
3	1.67	1.09	0.063	1.153	
4	1.67	1.33	0.045	1.375	
5	1.67	1.40	0.061	1.461	
6	1.67	1.52	0.085	1.605	
7	1.67	1.52	0.065	1.585	
8	1.67	1.52	0.088	1.608	
9	1.67	1.27	0.089	1.359	
	15.03	12.61	0.775	13.385	+1.645
Daily average.....	1.67	1.40	0.086	1.487	+0.18
February. <i>Bran ration + 6.08 grams of MgO as MgSO₄.</i>					
10	1.67	1.35	0.291	1.641	
11	1.67	1.42	0.093	1.513	
12	1.67	1.35	0.406	1.756	
13	1.67	2.03	0.720	2.750	
14	1.67	1.58	0.371	1.951	
15	1.67	1.34	0.299	1.639	
16	1.67	1.22	0.341	1.561	
	11.69	10.73	2.521	12.811	-1.121
Daily average.....	1.67	1.47	0.360	1.830	-0.160
<i>Bran ration + 6.08 grams of MgO as MgSO₄ + K₂HPO₄ to form Mg₃(PO₄)₂.</i>					
17	1.67	1.66	0.340	2.000	
18	1.67	1.34	0.200	1.540	
19	1.67	1.67	0.114	1.784	
20	1.67	1.44	0.108	1.548	
21	1.67	1.83	0.115	1.945	
22	1.67	1.50	0.115	1.615	
23	1.67	1.65	0.180	1.830	
24	1.67	1.35	0.096	1.446	
25	1.67	1.26	0.121	1.381	
26	1.67	1.53	0.123	1.653	
27	1.67	1.95	0.128	2.078	
	18.37	17.18	1.640	18.820	-0.450
Daily average of last nine days.....	1.67	1.57	0.122	1.697	-0.027

The data presented in the table show conclusively that a large amount of bran in the ration exerted no inhibiting action upon the animal's ability to assimilate lime from the ration. It is true that the amount retained was not very large; but, in any case, with the large losses normally occurring with the fecal excretion such a positive retention is to be viewed as a reliable index. With bran the bulk of the fecal excretion is large, which in itself may exert an intensified depreciating effect on the actual amount of lime absorbed from the ration itself.

The addition of magnesium salts did not increase the fecal lime excretion. Accompanied by the simultaneous addition of potassium phosphate a slightly increased lime content of the feces was observed. This cannot be overlooked as there is a possibility that the ingestion of the potassium phosphate may merely have changed the path of excretion for the lime. That this is not entirely the case is seen by the decrease in the total lime elimination during this period.

In the urine, with each period where magnesium salts alone were added, a remarkable increase in the urinary lime excretion is observed. This can be attributed to no other factor than magnesium salts, as the data from day to day are exceedingly consistent. The addition of soluble phosphates reduces this increment but not to its former level. Of the exact relations existing here the data offer us no information. But, in itself, it is truly remarkable that potassium phosphate should be able to counteract, even in part, the injurious action of the magnesium salts. Further, during this period there was not an increased magnesium excretion through the intestine.

Here, in the interrelations prevailing among mineral elements, we may have an explanation of some of the numerous anomalies that have been observed in connection with mineral metabolism experiments, otherwise well controlled. Though the effect of organic nutrients upon mineral metabolism is not to be underestimated, it undoubtedly is true that, in the condition of the digestive system and in the interrelations prevailing among mineral elements, we have two factors of primary importance operative in mineral metabolism.

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CONCLUSIONS.

1. The unfitness of bran as a bone producing feeding stuff is to be attributed to its low content of lime capable of absorption, rather than to a disproportionate content of calcium to magnesium.

2. Magnesium salts added to a pig's ration increased the calcium elimination in the urine. The fecal calcium excretion was not influenced.

3. Soluble phosphates, as di-potassium acid phosphate, decreased the increased calcium excretion brought on by the addition of magnesium salts.

4. The fact that the relation of phosphorus to calcium and magnesium in our grains is high, with the probable formation in the tract of magnesium phosphate and its excretion by way of the intestine, would help to explain the difference in the action of magnesium chloride or sulphate and the magnesium normal to grains.

5. The interrelations existing between mineral elements are important factors for consideration in studying the specific rôle of a mineral element in animal nutrition.

ON THE EXTREMES OF VARIATION OF THE CONCENTRATION OF IONIZED HYDROGEN IN HUMAN URINE.

By LAWRENCE J. HENDERSON AND WALTER W. PALMER.¹

(From the Chemical Laboratory, Massachusetts General Hospital.)

(Received for publication, January 20, 1913.)

In a recent paper² we have shown that the variations of hydrogen ion concentration in urine, when studied in a large number of instances, are not without physiological and pathological significance, and it is evident that the latitude of variation of this quantity is a fundamental factor in the regulation of the neutrality of the animal body. For this and other reasons, it is of interest to determine the extreme variations of acidity and alkalinity which can arise in man.

Considering the large amount of acid which is daily removed from the body (equivalent to 600–700 cc. of $\frac{N}{10}$ acid) and the enormous quantities which may be excreted in diabetic acidosis (equivalent to 6 liters of 0.1 N acid or more) it is obvious that higher acidity than that which is to be observed among the cases of a large general hospital is not to be expected under any circumstances, for it is not possible safely to introduce into the body more than a small fraction of such quantities of acid. Experiment bears out this view.

To study the effect of acid ingestion on the hydrogen ion concentration, 10 grams of monosodium phosphate were given at a single dose and the urine collected in separate specimens at frequent intervals thereafter.

The variation in hydrogen ion concentration from hour to hour in the various cases is only slightly greater than that which occurs in normal cases without monosodium phosphate intake. In every case, however, there is found a slight increase of hydrogen ion concentration. The greatest difference is observed in case 7,

¹ Henry P. Walcott Fellow in Clinical Medicine, Harvard Medical School.

² L. J. Henderson and W. W. Palmer: this *Journal*, xiii, p. 393, 1913.

Variations in Urinary Acidity

TABLE I.

*Concentrations of ionized hydrogen after the administration of acid phosphate.**

NUMBER	TIME ACID SODIUM PHOSPHATE GIVEN	TIME OF COLLECTION AND HYDROGEN ION CONCENTRATION OF URINE AFTER NaH ₂ PO ₄ INGESTION										MORNING FOLLOWING
		7.00 a. m.	9.00 a. m.	10.00 a. m.	11.00 a. m.	12.00 noon	1.00 p. m.	2.00 p. m.	3.00 p. m.	6.00 p. m.	10.00 p. m.	
	a. m.											
1	9.00		6.85	6.50	6.00	6.15		6.50	7.00		6.50	6.30
2	9.00		5.85	5.60	5.70	5.85	5.40	5.30	6.50			
3	9.00		5.40	5.70	5.85	5.30	5.15	5.30	5.60		5.70	
4	9.00		5.15	5.30	5.00		5.15	5.15		5.60	5.70	7.00
5	7.30	5.60			5.30				5.70	6.50	5.70	5.30
6	7.00	5.60							5.30	5.70	6.30	5.00
7	9.00		6.70	7.00	6.30	6.15	5.30	5.40				6.30
8	7.00	5.50					5.30			5.50	5.70	5.40

* Expressed as negative logarithms, see Henderson and Palmer, *loc. cit.*

where the initial value was low, 6.70, and at 1.00 p.m., four hours after the ingestion of monosodium phosphate, it was 5.30, representing a twenty-five fold increase in acidity. Administration of larger quantities of acid phosphate, or of hydrochloric acid, produces similar effects and never, in our experience, causes acidity of the urine as great as that which is common in many pathological conditions.

On the other hand the body is not known ever to produce bases in considerable quantity (except ammonia to neutralize an excess of acid) hence it is natural to expect that the administration of alkali in the normal individual may produce a urine more alkaline than that which is otherwise to be found. This view also has been confirmed by experiment.

TABLE II.

NUMBER	+ H WITHOUT ALKALI	FIRST DAY WITH ALKALI		SECOND DAY WITH ALKALI	
		Sodium bicarbonate	+ H	Sodium bicarbonate	+ H
		grams		grams	
1	6.15	24	8.00	40	8.70
2	6.00	20	8.70		
3	6.30	12	7.40		
4	6.70	20	8.30		
5	6.00	24	8.30	40	8.70
6	6.85	8	8.50	16	8.50
7	6.50	12	8.30	24	8.50
8	6.70	12	8.70		

The effect of the ingestion of large amounts of sodium bicarbonate was studied in young adults. As far as possible the sodium bicarbonate was taken between meals and the hydrogen ion concentration was determined on twenty-four-hour specimens of urine.

The results of a single dose of sodium bicarbonate on the hydrogen ion concentration are given in the following table. As far as possible a specimen of urine was obtained at the time of administration of the sodium bicarbonate and hourly specimens obtained for several hours thereafter.

TABLE III.

NUMBER	SODIUM BICARBONATE	TIME SODIUM BICARBONATE GIVEN	TIME OF COLLECTION OF SPECIMEN OF URINE AND HYDROGEN ION CONCENTRATION					
			10.00 a. m.	11.00 a. m.	12.00 noon	1.00 p. m.	2.00 p. m.	3.00 p. m.
	<i>grams</i>	<i>a. m.</i>						
1	4	10.00	7.40	8.30	7.48	7.48	7.40	5.85
2	8	10.00	5.40	8.50	8.30	6.50	6.50	7.40
3	12	10.00	5.30	8.70	8.70	8.70	8.70	8.70
4	8	10.00	7.40	8.50	8.70	8.50	8.50	8.50
5	8	10.00	5.85			8.70	8.70	8.30
6	8	10.00	6.70	7.48	8.70	8.50	8.70	8.50

In no instance were we able, by the administration of sodium bicarbonate, to obtain a more alkaline urine than one with a hydrogen ion concentration of 8.70, which is slightly more alkaline than a solution of disodium phosphate of concentration about 0.01 N. From the foregoing data it is evident that the reaction of the urine may be pushed down to a certain degree of alkalinity with comparative ease; beyond this point, even after the administration of large amounts of alkali, the reaction of the urine does not change.

The highest acidity which has thus far been observed, exceeds 4.70; the highest alkalinity, 8.70. This corresponds to a range of 1 : 10,000 in the concentration of hydrogen and hydroxyl ions, and amounts on the one hand to the acidity of a solution consisting of a trace of free phosphoric acid together with monosodium phosphate, on the other hand, to the alkalinity of a solution of disodium phosphate of concentration about 0.01 N.

The variations of the relative amounts of acid and alkali which accompany such variations in reaction are very large and, together with ammonia excretion, measure the protective power of the kidney.

Ten cubic centimeter samples from twenty-four-hour amounts of urine from normal individuals and 10 cc. of a standard solution having a hydrogen ion concentration of 4.70 (highest observed acidity) were introduced into separate 250 cc. flasks, diluted with distilled water to approximately 250 cc. and five drops of a 2 per cent aqueous solution of sodium alizarine sulphonate added. Sufficient sulphuric acid of concentration 0.1 N was added to the flask containing urine to bring its reaction to that of the standard solution. A second titration was carried out with a fresh 10 cc. sample of urine and 10 cc. of a standard solution with a hydrogen ion concentration of 8.70 (lowest observed alkalinity), similarly diluted, ten drops of a 1 per cent alcoholic solution of phenolphthalein added, and the reaction of the urine carried to that of the standard solution by addition of potassium hydrate of concentration 0.1 N. In each instance the number of cc. of $\frac{N}{10}$ acid or alkali to bring the reaction of the total amount of urine to the reaction of the standard solutions was calculated.

TABLE IV.

NUMBER	AMOUNT	+ H	TOTAL ACID	TOTAL ALKALI	ACID + ALKALI	RATIO OF ACTUAL TO POS- SIBLE ALKALI IN URINE
	cc.		cc.	cc.	cc.	
1	2200	6.15	185	246	431	0.43
2	1200	6.30	380	568	948	0.40
3	1300	6.30	284	545	829	0.34
4	1300	5.85	285	705	990	0.29
5	1500	7.00	411	212	623	0.61
6	2500	6.70	500	680	1180	0.43
7	2175	6.50	425	675	1100	0.39
8	1000	5.85	188	385	573	0.33
9	1920	6.85	432	430	862	0.50
10	1300	6.70	271	316	587	0.45
Average (alkali + acid).....						812
Maximum (alkali + acid).....						1180
Minimum (alkali + acid).....						431

It thus appears that the possible variation in the amount of alkali in union with the acids of normal urine is ordinarily about 0.5–1.0 gram-molecule. In pathological conditions, when the acids are increased, this quantity also is necessarily larger.

The actual variation in normal individuals is evidently at least nearly 0.5 gram-molecule.

In the most acid urines which we have observed, the urinary acids, in addition to phosphoric acid and acid sodium phosphate, are free to a considerable degree, as follows: hippuric acid, 8 per cent, acetoacetic acid, 11 per cent; lactic acid, 12 per cent; β -oxybutyric acid, 45 per cent; uric acid, 91 per cent.

In the most alkaline urines all these acids are almost completely combined with base, and any free carbonic acid must be accompanied by a very large quantity of sodium bicarbonate—at least one hundred times its concentration. Such is the explanation of the large variation in relative amounts of acid and alkali.

We wish to thank Dr. Frederick C. Shattuck for making this work possible. Thanks are also due to the members of the visiting staff and others of the Massachusetts General Hospital for their kindness in supplying clinical material.



THE ESTIMATION OF CREATININE AND CREATINE IN DIABETIC URINES.

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(Received for publication, January 21, 1913.)

That acetone and acetoacetic acid react with picric acid and sodium hydroxide with the production of a colored solution, similar to that produced by creatinine under the same conditions, has long been known. The extent of their possible interference with the estimation of creatinine, by the Folin method, has not been clearly determined. Folin¹ states that the reaction is given by hydrogen sulphide, acetone, acetoacetic acid and its ester, which, if present, may easily be removed. No directions are given. Klercker,² who used very large amounts of acetone in his experiments, found that acetone caused a rapid fading of the color. According to van Hoogenhuyze and Verploegh³ the readings obtained in the presence of acetone are at first too low, but as the color due to the acetone fades rapidly, normal readings are soon obtained. Similar results are reported by Krause,⁴ who also investigated the action of acetoacetic acid. He found that the readings were too low. Wolf and Osterberg,⁵ on the contrary, reported that the addition of 1 per cent of acetoacetic ester was without influence upon the determination of creatinine. Rose⁶ used acetoacetic acid in concentrations up to 0.25 per cent and obtained correct readings if the solutions were allowed to stand three or four minutes after dilution before making the readings.

¹ Folin: *Zeitschr. f. physiol. Chem.*, xli, p. 223, 1904.

² Klercker: *Biochem. Zeitschr.*, iii, p. 45, 1907.

³ van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 161, 1908.

⁴ Krause: *Quart. Journ. Exp. Physiol.*, iii, p. 289, 1910.

⁵ Wolf and Osterberg: *Amer. Journ. of Physiol.*, xxviii, p. 71, 1911.

⁶ Rose: *this Journal*, xii, p. 73, 1912.

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In connection with an investigation into the creatine and creatinine metabolism in diabetes, it became necessary to ascertain if acetone and acetoacetic acid in the amounts present in diabetic urines would interfere with the estimation of creatinine. This was found to be the case. Although the addition of a small quantity of acetone (0.5 per cent) to normal urine does not greatly affect the readings obtained, such addition to diluted urine or to urine containing but little creatinine, interferes very decidedly. The presence of acetoacetic acid also makes all the readings unreliable. The figures given in Table I are typical of the results obtained.

TABLE I.

The effect of acetone and acetoacetic acid upon the estimation of creatinine.
(10 cc. of the urine were diluted to 250 cc. after the addition of the picric acid and sodium hydroxide solutions.)

NUMBER	NATURE OF URINE	ADDITION	READINGS
1	Normal, diluted		11.06
		0.5 per cent acetone	10.30 fading to 11.6
2	Normal, diluted	Acetoacetic acid*	12.00 fading to 12.4
			10.10
3	Muscular dystrophy	Acetoacetic acid*	10.80 fading to 13.0
			10.50
		Acetoacetic acid*	13.00

* Enough of a freshly prepared solution of acetoacetic acid was added to produce a Gerhardt reaction comparable to that given by a urine then under examination in this laboratory.

In order to make an accurate estimation of creatinine, by the Folin method, it is therefore necessary either to remove the interfering substances or to precipitate the creatinine, determine the creatine in the filtrate and subtract the amount found from the sum of the creatine and creatinine. Boiling the urine, as recommended by Rona,⁷ was first attempted. It was found that the urine from a severe case of diabetes required boiling for at least five minutes to free it of acetoacetic acid. A number of experiments were made with urines from patients with muscular dystrophy. Creatinine was determined in the usual manner. A volume of urine equal to that taken for the determination of creatinine was boiled for five minutes, cooled and the creatinine estimated. In every instance the readings were much lower than

⁷ Rona: *Handbuch d. biochem. Arbeitsmethoden*, iii, p. 788, 1910.

TABLE II.

The effect of boiling the urine upon the estimation of creatinine in the presence of creatine.

NUMBER	VOLUME OF URINE	DILUTION	READINGS	
			Unboiled	Boiled
	cc.	cc.	mm.	mm.
1	25	500	6.87	5.20
2	25	1000	8.13	6.97
3	25	250	7.23	6.12
4	25	300	8.55	7.35

in the control estimation (Table II). The method was therefore rejected.

Fairly good results were obtained by precipitating the creatinine with sulphuric and phosphotungstic acids, filtering, freeing an aliquot portion of the filtrate of the acids with barium hydroxide, filtering, precipitating the excess of barium in an aliquot portion of the filtrate with sodium sulphate, adding hydrochloric acid, evaporating and completing the determination of creatine in the usual manner. A urine from a case of muscular dystrophy contained 0.276 gram preformed, and 0.881 gram total creatinine per liter. To some of this urine, 4 per cent of dextrose was added and the determination of creatine carried out as described above. Calculated as creatinine, 0.611 gram was found. In another urine the content of preformed creatinine was 0.822 gram and of total creatine, 1.200 grams per liter. The amount of creatine, as estimated by this method after the addition of 4 per cent of dextrose to the urine, was 0.382 gram, calculated as creatinine, per liter. As a rule, however, the agreement was not so close as in these instances. The method is not altogether satisfactory.

At the suggestion of Dr. S. R. Benedict, an attempt was made to determine the creatinine after extraction of the acetoacetic acid with ether and subsequent aeration to remove the ether and acetone. This was found to be very successful. The apparatus used consisted of an ordinary Soxhlet extraction apparatus, a short, wide test-tube and a funnel-tube. In principle it was the same as that described by Saiki.³ After two hours the urines were found to be free of appreciable amounts of acetoacetic acid. For

³ Saiki: this *Journal*, vii, p. 21, 1909.

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the determination of creatinine the following procedure was adopted: 25 cc. of the urine are measured into the extraction tube, 1.0 cc. of concentrated hydrochloric acid added and the mixture extracted with ether for two hours. The contents of the tube are then washed into an aerometer cylinder and aerated for one hour. To reduce foaming, a few drops of toluene may be added and the aeration continued until this has been removed. The estimation is then carried out in the usual manner except that, because of the dilution, 30 cc. of the picric acid solution and 10 cc. of the sodium hydroxide solution are used. Sodium hydroxide equivalent to the hydrochloric acid used must also be added. The results of a number of estimations in urines from patients with muscular dystrophy are summarized in Table III. In order to eliminate the influence of suggestion, most of the determinations were made by two observers, one comparing the colors, the other reading the scale.

That dilute hydrochloric acid does not, under these conditions, effect an appreciable conversion of creatine into creatinine is also

TABLE III.

Comparison of results obtained by direct estimation of creatinine in urines containing creatine and after addition of acetoacetic acid and its extraction with ether.

NUMBER	DIRECT	AFTER ADDITION OF ACETOACETIC ACID AND EXTRACTION	TIME ELAPSED BETWEEN THE ADDI- TION OF THE HYDRO- CHLORIC ACID AND THE ESTIMATION
	mgm. per liter	mgm. per liter	hours
1	235	235	
2	226	229	
3	472	472	
4	793	797	4
5	613	610	
6	232	228	3
7	494	486	6
8	494	498	18
9	434	432	3.5
10	434	439	8
11	434	434	4
12	395	398	4
13	424	430	20
14	541	537	5
15	359	359	7

indicated by the following experiments. Forty-eight milligrams of creatine were dissolved in 100 cc. of 0.5 N HCl solution. At intervals the liquid was tested for creatinine by Jaffé's reaction and also with phosphotungstic acid. Even after four days there was no precipitation on the addition of phosphotungstic acid nor was the color produced with picric acid and sodium hydroxide different from that of the alkaline picrate solution. On another occasion, 0.119 gram of creatine was dissolved in 100 cc. of water and 4 cc. of concentrated hydrochloric acid. After six hours at a temperature between 37° and 40°, the solution gave no precipitate with phosphotungstic acid and only a slight reaction with picric acid and sodium hydroxide solutions.

TABLE IV.

Comparison of readings obtained in the direct estimation of creatinine in diabetic urine and after extraction with ether.

(25 cc. of urine diluted to 250 cc. before reading.)

NUMBER	DIRECT	AFTER EXTRACTION
	mm.	mm.
1	9.3	7.80
2	9.4	8.30
3	14.7	9.20
4	11.1	7.12
5	11.6	6.86

A few examples of the results obtained by this method, as compared with the original Folin method, are given in Table IV. The urines were obtained from a patient with a severe form of diabetes. The figures in the first column were obtained after waiting until the readings no longer changed appreciably within three or four minutes. This required from five to ten minutes. The color may continue to fade slowly for a much longer time as is shown by two experiments, the results of which are given in Table V. The readings did not remain constant until about thirty minutes had elapsed.

Rose⁹ has called attention to the low readings obtained, in the presence of dextrose, in the Benedict-Myers modification of the Folin method for the determination of creatine. He has proposed

⁹ Rose: *loc. cit.*

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the substitution of phosphoric acid for the hydrochloric acid. Apparently he did not use the original Folin method. As is shown in Table VI, this is quite satisfactory. If care be taken to prevent undue concentration, the effect of dextrose, even in a concentration of 5 per cent, is barely appreciable, although the urines contain so little creatinine that the dilution before reading is only 250 or 300 cc.

TABLE V.

Readings obtained in the estimation of creatinine in diabetic urine by the Folin method.

	I	II		I	II
Immediately.....	13.0	6.9	After twenty minutes...	18.2	10.2
After five minutes.....	14.7		After thirty minutes...	19.0	10.2
After ten minutes.....	16.8	9.0	After forty-five minutes	19.0	
After fifteen minutes...	18.0	9.3			

TABLE VI.

The effect of dextrose upon the estimation of creatine by the Folin method. (10 cc. of diluted normal urine made up to 250 or 300 cc. before reading.)

NUMBER	DEXTROSE ADDED	READINGS	NUMBER	DEXTROSE ADDED	READINGS
	gram	mm.		gram	mm.
1		9.00	5		7.93
	0.3	8.95		0.5	7.80
2		6.88	6		8.07
	0.4	6.83		0.5	7.98
3		5.57	7		9.90
	0.5	5.45		0.5	9.74
4		7.75	8*		7.01
	0.5	7.80		0.5	7.08

* Dog urine.

In this laboratory, the conversion of creatine into creatinine is generally accomplished in 50 cc. flasks with short necks. The condensation in the neck prevents the loss of any appreciable amount of water but the acetone is almost completely removed. Three urines, which contained, respectively, 14.6, 10.5 and 17.6 mgm. of acetone in 10 cc., after being heated with hydrochloric acid as in the determination of creatine, contained only 1.7, 1.0

and 1.9 mgm. of acetone in 10 cc. Such small amounts of acetone are without appreciable effect on the readings obtained.

The Folin method is so simple that it seems to be preferable to the much more troublesome procedure recommended by Rose. The only advantage of the latter is that conversion is completed a little sooner, but the time actually required for the manipulation is greater. Except when a large number of determinations are to be made at the same time, it offers no advantages over the Folin method.

SUMMARY.

In urines containing acetoacetic acid or acetone, creatinine may be accurately estimated by the Folin method only after removal of these substances. A method of accomplishing this is described. Dextrose, in concentrations up to 5 per cent, is without appreciable effect upon the estimation of creatine by the Folin method.



ON THE COLORIMETRIC DETERMINATION OF URIC ACID IN URINE.

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By means of the new colorimetric method for the determination of uric acid in urine recently described by Folin and Macallum¹ it is possible to make a reliable uric acid determination in about fifteen minutes. The method consists essentially in evaporating the acidified urine to dryness and extracting from the residue certain polyphenol compounds which give a blue reaction with the uric acid reagent.² The insoluble uric acid is left behind and is then determined colorimetrically. Unfortunately this method is not directly applicable to all kinds of urine. Urines of certain animals as for example the rat and cat and also certain kinds of pathological human urine (urines containing albumin or sugar) leave on evaporation such coatings of inert materials that it is practically impossible to remove quantitatively the interfering polyphenol compounds by means of alcohol-ether mixtures or indeed by any other solvent which does not also dissolve uric acid.

In their preliminary paper Folin and Macallum³ described a different method for separating the uric acid from the disturbing polyphenols. The uric acid was precipitated by means of ammo-

¹ This *Journal*, xiii, p. 363, 1912.

² The urine was acidified with oxalic acid before the evaporation. This produced some urea oxalate which protected the uric acid mechanically without interfering appreciably with the alcohol-ether extraction. Recently C. Farmer and F. B. Grinnell have made a series of extractions after acidifying with 1 cc. of 1 per cent monosodium phosphate solution. Judging from the results obtained with pure uric acid solutions as well as with normal urines the results so obtained are rather more satisfactory with pure uric acid solutions and equally satisfactory with urine.

³ This *Journal*, xi, p. 265, 1912.

niacal silver solutions more or less as in the Ludwig-Salkowski method. The great drawback to this procedure was the fact that in the presence of the silver the blue color obtained on adding the uric acid reagent was so very transient in character that correct results could be obtained only by working with a very high degree of speed and uniformity of procedure.

Having solved the problem of how to make use of ammoniacal silver solution as a reagent for isolating uric acid from blood in a condition suitable for colorimetric determinations⁴ we had, of course, in that procedure one that could hardly fail to be applicable to all kinds of urine. The general method thus evolved necessitates the use of a small centrifuge but it is otherwise as rapid, convenient and accurate as the method described by Folin and Macallum for normal urine. At all events because of its general applicability to pathological urines the method would seem to merit a separate description, especially as we are now able also to describe a method for the preparation of a satisfactory standard.

Standard uric acid solutions for the colorimetric determination of uric acid.

No other detail in the colorimetric determination of uric acid has presented such difficulties as has the problem of finding a suitable standard. Theoretically the most satisfactory standard for any color comparison is the color obtained with a known amount of the substance which is to be determined. In all our work we have used fresh uric acid solutions for this purpose because no other stable substance has been found to give exactly the same color as that given by uric acid. It is distinctly tedious, however, to make up standard uric acid solutions every day or every few days. The standard which was obtained by adding an excess of uric acid to an exact amount of the uric acid reagent⁵ was not entirely satisfactory, the chief drawback being the distinctly greater rapidity with which the color of such solutions fade as compared with the solutions obtained by adding an excess of the reagent.

⁴ This *Journal*, xiii, p. 469, 1913.

⁵ This *Journal*, xiii, p. 367, 1912.

By combining uric acid with formaldehyde we have at last obtained solutions of uric acid which act like ordinary pure uric acid solutions with reference to the quality and stability of the color produced with an excess of the uric acid reagent. The solutions so obtained seem to keep their strength indefinitely. We have now had several different solutions for three months, have kept them in the light and in the dark, in cold places and in warm ones, and in no case have we observed any diminution in the color value obtained with a given excess of the uric acid reagent. The uric acid formaldehyde compounds do not possess more than a small fraction of the reactivity corresponding to the uric acid contained in them and their value in terms of uric acid must be determined just like that of any unknown uric acid solution but the important thing is that the active fraction, whatever it is due to, remains constant.⁶

The uric acid formaldehyde solution is prepared as follows: One gram of uric acid in a volumetric liter flask is dissolved by means of an excess of lithium carbonate (200 cc. of a 0.4 percent solution). To the solution are added 40 cc. of 40 per cent formaldehyde solution and the mixture is shaken and allowed to stand for a few minutes. The clear solution is acidified by the addition of 20 cc. of normal acetic acid and the whole is diluted up to the liter mark with water. The solution should remain perfectly clear and the next day (but not before) it can be standardized against a freshly prepared lithium carbonate solution of uric acid. The color produced by 5 cc. of the solution corresponds very nearly to the color obtained from 1 mgm. of uric acid. The colorimeter reading obtained for the solution when thus compared against 1 mgm. of pure uric acid is, of course, thereafter to be used as the standard value corresponding to 1 mgm. of uric acid.

The new uric acid method.

From 1 to 2 cc. of urine are measured into an ordinary centrifuge tube by means of a modified Ostwald pipette. A sufficient amount of distilled water is then added to bring the volume of

⁶ Uric acid formaldehyde compounds were first prepared by Tollens (*Ber. d. deutsch. chem. Gesellsch.*, xxx, p. 2514, 1897). The solubility of these compounds has long been known but their stability in aqueous solutions has evidently not been recognized before.

the liquid in the tube to about 5 cc., six drops of 3 per cent silver lactate solution, two drops of magnesia mixture, and a sufficient amount (10-20 drops) of concentrated ammonium hydrate to dissolve the silver chloride are then added. The tube is now centrifuged for one or two minutes, the supernatant liquid poured off and to the residue in the bottom of the tube are added five or six drops of freshly prepared saturated hydrogen sulphide water and one drop of concentrated hydrochloric acid, and the tube is placed in a beaker of boiling water until all excess of hydrogen sulphide has been driven off.

As hydrogen sulphide gives a blue color with the "uric acid reagent" care must be taken to obtain its complete removal. To determine whether this has been accomplished one drop of 0.5 per cent lead acetate solution should be added to the contents of the tube after the latter has remained in the water bath for about five minutes and if any hydrogen sulphide still remains a dark brown precipitate will be formed. If this condition be obtained the tube should be returned to the water bath for a further period of heating.

When the tube has been cooled, add 2 cc. of the uric acid reagent, 10 cc. of saturated sodium carbonate solution, transfer to a 50 cc. volumetric flask and make up to volume. The color comparison is then made in the usual manner against the color obtained from 5 cc. of the standardized uric acid-formaldehyde solution (or a freshly prepared pure uric acid solution).

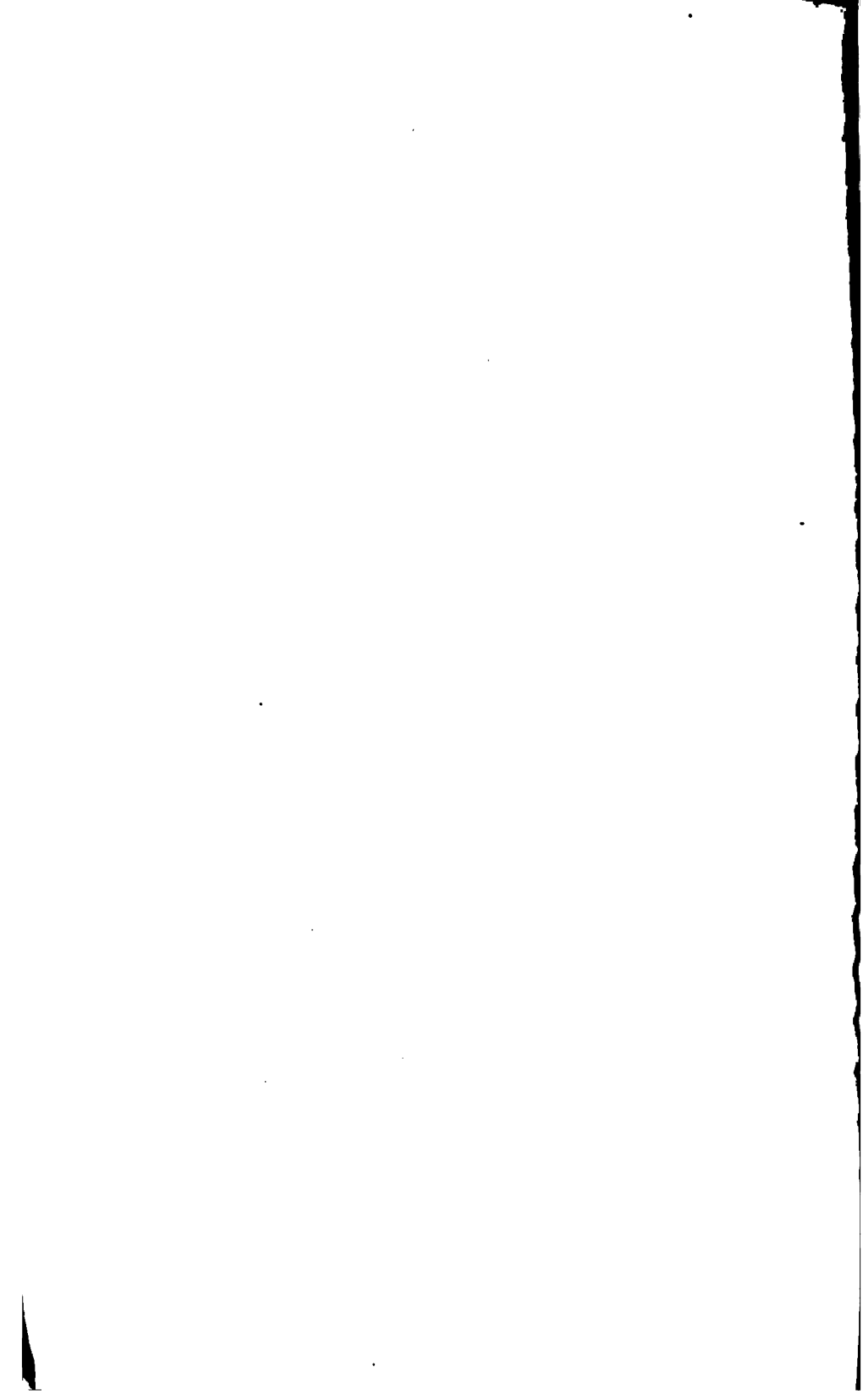
In the case of urines containing much albumin it will be found that after the addition of hydrogen sulphide the solution obtained is invariably of a brownish tint, which interferes with the color comparison, and thus makes accurate readings very difficult. This difficulty may be overcome by adding to the hot solution (after the removal of all hydrogen sulphide) from two to ten drops of a 10 per cent sodium acetate solution.

This procedure has also been found useful in the determination of uric acid in blood where the same trouble is met with when as occasionally happens the protein has not been entirely removed.

Unless albumin be present sodium acetate should not be added either in blood or urine analysis as its presence tends to give slightly low results.

The uric acid determinations recorded below show that the new method is capable of giving excellent results in the presence of large amounts of albumin or sugar. The figures represent grams of uric acid per liter of urine.

NO. OF URINE	FOLIN-SHAFTER METHOD	NEW METHOD	NO. OF URINE	FOLIN-SHAFTER METHOD	NEW METHOD
1.....	0.49	0.50	12.....	0.70	0.76
1 + 10% serum.....		0.52	12 + 20% serum.....		0.75
2.....	0.49	0.49	13.....	0.60	0.57
2 + 10% serum.....		0.50	13 + 5% dextrose.....		0.57
2 + 20% serum.....		0.47	13 + 10% dextrose.....		0.57
3.....	0.84	0.80	14.....	0.82	0.83
3 + 10% serum.....		0.82	14 + 5% dextrose.....		0.83
3 + 20% serum.....		0.80	14 + 10% dextrose.....		0.86
4.....	0.60	0.60	15.....	0.58	0.62
4 + 10% serum.....		0.59	15 + 5% dextrose.....		0.62
5.....	0.69	0.71	15 + 10% dextrose.....		0.62
5 + 10% serum.....		0.70	16.....	0.65	0.70
6.....	0.47	0.49	16 + 5% dextrose.....		0.70
6 + 40% serum.....		0.50	16 + 10% dextrose.....		0.69
7.....	0.16	0.17	17.....	0.68	0.71
7 + 40% serum.....		0.17	17 + 5% dextrose.....		0.70
8.....	0.47	0.44	17 + 10% dextrose.....		0.71
8 + 20% serum.....		0.45	18.....	0.47	0.46
9.....	0.65	0.67	18 + 5% dextrose.....		0.46
9 + 2% egg albumin.....		0.66	18 + 10% dextrose.....		0.47
10.....	0.44	0.42	19.....	0.87	0.82
10 + 20% serum.....		0.43	19 + 5% dextrose.....		0.80
11.....	0.48	0.50	19 + 10% dextrose.....		0.81
11 + 20% serum.....		0.49			



ON THE NATURE OF THE IODINE-CONTAINING COMPLEX IN THYREOGLOBULIN.

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of Chicago.)

(Received for publication, January 27, 1913.)

In this paper are given the results of an attempt to determine the nature of the active complex in the iodine-containing active principle of the thyroid gland. Although the nature of this group was not determined, the quantitative physiological results here reported serve to establish certain predicted and other unexpected facts and to eliminate certain hitherto considered probabilities.

The problem was taken up both by analytical and by synthetic methods. In the former method the physiological activity and iodine content of the dried thyroid tissue, the globulin therefrom and various products of hydrolysis from this globulin were determined quantitatively. In the second method two iodized amino-acid derivatives, not previously tested by quantitative methods, were prepared synthetically and their physiological activity studied quantitatively.

In thus tracing the active complex a number of important assumptions were made. First, that the activity of unaltered thyroid tissue depends quantitatively on its iodine content. Second, that the best method known for measuring this activity directly and quantitatively is the Reid Hunt acetonitrile test.¹ Third, that in case the iodine is present in the products of hydrolysis in the same combination as in the globulin then, per unit of iodine, these will still possess an activity comparable with the original globulin. Fourth, that in case the iodine complex is an iodized amino-acid and that in case this is decomposed in the process of hydrolysis then the synthetic preparation of various iodized amino-acids or derivatives thereof and the quantitative testing of

¹ This *Journal*, i, p. 33, 1905.

these per unit of iodine may determine the probable nature of the iodine complex. In other words, the actual quantitative physiological activity per unit of iodine as measured by the Reid Hunt method was taken as the crucial test for the presence or absence of the unaltered iodine complex.

The historical development of the relation of thyroid activity to iodine content need not be considered at this time, especially in view of the thorough reviews and extensive confirmatory experiments made by Reid Hunt and A. Seidell,² as well as the comparative histological and chemical studies by Marine in coöperation with Lenhardt and Williams.³ A careful study of these papers justifies the first assumption. The second assumption is also well taken provided the proper precautions are observed as shown by Reid Hunt and A. Seidell.⁴ Other methods for testing the physiological activity of thyroid substance, based on changes in blood pressure,⁵ on increasing the irritability of the depressor nerve,⁶ on changes in nitrogen metabolism⁷ and on curative effects in cretinism⁸ have been employed, but are not applicable in a quantitative study, nor are they as specific reactions.

Of the third and fourth assumptions we had no definite proof. The studies of Oswald⁹ and others show that during hydrolysis of thyreoglobulin only 30 per cent or less of the iodine remains in organic combination. The iodine thus combined is in the various fractions and qualitatively it has been determined¹⁰ that probably the greater activity remains in the more complex products

² Bulletins 47 (1908) and 69 (1910) of the Hygienic Laboratory, U. S. Public Health and Marine Hospital Service.

³ *Johns Hopkins Hospital Bull.*, xviii, p. 359, 1907; *Journ. Inf. Dis.*, iv, p. 417, 1907; *Archives of Internal Med.*, i, p. 349, 1908; *Ibid.*, iii, p. 66, 1909; *ibid.*, iv, p. 440, 1909; *ibid.*, vii, p. 506, 1911; *ibid.*, viii, p. 265, 1911; *Journ. of Exp. Med.*, xiii, p. 455, 1911.

⁴ *Loc. cit.*; *Journ. of Pharmacol. and Exp. Ther.*, ii, p. 15, 1910.

⁵ von Fürth and Schwarz: *Pflüger's Archiv*, cxxiv, p. 113, 1908.

⁶ von Cyon and Oswald: *Pflüger's Archiv*, lxxxiii, p. 199, 1901; Asher and Flack: *Zeitschr. f. Biol.*, lv, p. 83, 1910.

⁷ Baumann: *Zeitschr. f. physiol. Chem.*, xxi, p. 487, 1896; *ibid.*, xxii, p. 1, 1896; *Münch. med. Wochenschr.*, xl, 1896.

⁸ E. Pick and F. Pineles: *Zeitschr. f. exp. Path. u. Ther.*, vii, p. 518, 1909-10.

⁹ *Arch. f. exp. Path. u. Pharm.*, lx, p. 115, 1908.

¹⁰ Pick and Pineles: *loc. cit.*

of hydrolysis where also the greater part of the organically combined iodine is found. What relation the activity bears to the iodine content therein has however not been determined. As stated above we have evidence that some of the iodine is split off as iodide, but we have no direct evidence that all the organically combined iodine found in the products of hydrolysis is still in the same complex or in the same structural relationship as in the original thyreoglobulin. A number of iodized amino-acids have been studied qualitatively as to physiological activity. In no case has thyroid activity been detected. The most conclusive results as to the inactivity of 3,5-iodo-laevo-tyrosine are those reported by Strouse and Voegtlin.¹¹ Other observations on the inactivity of various iodized proteins, which on hydrolysis yield 3,5-iodo-tyrosine, also bear out these conclusions. The studies on other iodized amino-acids do not lead to definite conclusions. Thus von Fürth and Schwarz¹² prepared and studied what they considered iodized phenylalanine, histidine and tryptophane. They reported all these substances as physiologically inactive, but gave no data indicating that they had really separated iodo-derivatives of these substances. Pauly¹³ however actually separated pure tetra-iodohistidine anhydride and tri-iodo-imidazol and reported that these substances increased the respiratory and pulse frequencies, although uniodized imidazol had no such action. These considerations lead us to conclude that for the present the validity of the third and fourth assumptions is unknown to us and that the true answers thereto are part of the problem in hand.

EXPERIMENTAL PART.

The mode of attack has already been outlined above. The details as to the methods employed and the preparation of the substances studied are given below.

A. Preparations.

Dried hog thyroids. Hog thyroids¹⁴ were freed mechanically from fat as much as possible and dried on glass plates in a current of air at 30-35°C.

¹¹ *Journ. of Pharm. and Exp. Ther.*, i, p. 123, 1909.

¹² *Pföüger's Archiv*, cxxxiv, p. 113, 1908.

¹³ *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 2243, 1910.

¹⁴ The raw material for this research was supplied by the Armour Laboratory Department.

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The mass was then ground to a coarse powder and fat removed by ether in the cold. The remaining dry mass was then finely powdered. Duplicate determinations on this gave 0.243 and 0.250 per cent iodine.

Thyreoglobulin. This was prepared as previously described.¹² Duplicate determinations on this gave 0.462 and 0.468 per cent iodine.

Iodothyryn (a) was prepared by the usual Baumann process from the above thyreoglobulin. The extraction with 95 per cent alcohol of the melanoidin precipitate was made in a continuous hot extractor. Duplicate determinations gave 5.81 and 5.85 per cent iodine.

Iodothyryn (b) was obtained in the same way from the melanoidin precipitate which separated in the complete hydrolysis of some of the same thyreoglobulin by 30-35 per cent sulphuric acid. This on analysis gave 7.51 per cent iodine.

Iodothyryn (c) was obtained from 40 grams of the same globulin by hydrolysis for three days at room temperature and for twenty-four hours at boiling temperature with 20-25 per cent phosphoric acid. Phosphoric acid was used as it was thought that possibly the oxidative action of sulphuric acid might have an injurious effect. This amount of globulin yielded 3.30 grams of melanoidin, containing 1.75 per cent iodine. The iodothyryn extracted from this represented 24 per cent of the weight and contained 4.44-4.46 per cent iodine. Thus only 61 per cent of the iodine in the melanoidin fraction was recovered in the alcohol extract.

Metaprotein (A₄). The filtrate from the melanoidin fraction above was neutralized with NaOH and the metaprotein separated and dried over sulphuric acid in a vacuum desiccator. This weighed 1.62 grams and contained 1.51-1.53 per cent iodine.

Primary albumose (A₅). The filtrate from above was half saturated with zinc sulphate after slightly acidifying with sulphuric acid. The precipitate obtained was dialyzed until free from sulphate. In this fraction there were recovered 3.1 grams containing 0.22-0.225 per cent iodine.

Secondary albumose (A₆). Obtained from the filtrate from above by complete saturation with zinc sulphate. The precipitate after dialyzing as above yielded 4 grams dry substance containing 0.069 per cent iodine.

The table (I) below gives a summary of the distribution of iodine in the different fractions above.

TABLE I.

	WEIGHT RECOVERED	PER CENT OF IODINE THEREIN	WEIGHT OF IODINE	PER CENT OF TOTAL IODINE IN THE GLOBULIN
Melanoidin precipitate	3.30	1.74	0.0575	30.9
Metaprotein.....	1.62	1.52	0.0246	13.2
Primary albumose.	3.01	0.22	0.0066	3.5
Secondary albumose.	4.0	0.0695	0.0027	1.5
Undetermined iodine.				50.9

¹² This *Journal*, ix, p. 121, 1911.

Phosphotungstic acid precipitate. Another 40 grams of thyreoglobulin were boiled with 25 per cent phosphoric acid for ninety-three hours. The filtrate from the melanoidin precipitate and metaprotein, after removal of the phosphoric acid by $\text{Ba}(\text{OH})_2$ and the excess of barium by sulphuric acid, was concentrated under diminished pressure to about 250 cc. This was then freed from proteose and peptone by the Kutscher tannin method.¹⁶ The filtrate finally obtained here after removal of the excess of lead was boiled with BaCO_3 to remove the ammonia. The dissolved barium was again removed by sulphuric acid. The filtrate after acidifying with H_2SO_4 to 5 per cent strength was precipitated with phosphotungstic acid in the usual way. The precipitate after thorough washing with 2.5 per cent phosphotungstic acid solution was freed from phosphotungstic acid, barium and sulphate in the usual way. Duplicate determinations on the dry amino-acid mixture gave 0.0107 per cent and 0.0093 per cent iodine.

Another phosphotungstic acid precipitate from a hydrolysis by H_2SO_4 was worked up in the same way. This dry residue contained 0.0068 per cent iodine. The two samples were mixed and designated as P.T.A. Ppt. 1. This mixture contained 0.0073 per cent iodine.

Phosphotungstic acid filtrate (1). This was freed from phosphotungstic acid in the usual way. The amino-acid solution was evaporated to dryness. Duplicate determinations on the dry amino-acid mixture gave 0.0024 per cent iodine.

Phosphotungstic acid precipitate (2). This was obtained in the same way as the above from the partial hydrolysis by 10 per cent sulphuric acid of 141.6 grams of thyreoglobulin containing 0.511 per cent iodine. The purified dry residue by analysis contained 0.0043 per cent iodine.

Phosphotungstic acid filtrate (2). The filtrate from the above was treated in the usual way. The dry purified amino-acid mixture left gave in duplicate determinations 0.0045 and 0.0043 per cent iodine.

Tetra-iodohistidine anhydride. Histidine was prepared from ox erythrocytes by the method of Frankel.¹⁷ Various methods were employed in trying to iodize the dichloride or the base itself but in no case were there indications of true absorption of iodine, but rather decomposition of the histidine. While this work was under way Pauly¹⁸ published his observations with the same conclusions as to the difficulty or inability to iodize histidine directly. At the same time, as stated above, he published his observations on tetra-iodohistidine anhydride. Following the methods given by Pauly¹⁹ the preparation of the methyl ester of histidine dichloride was carried out and from this the histidine anhydride by the Pauly modification²⁰ of the Fischer and Zuzuki method. The histidine anhydride was recrystallized from hot water a number of times to obtain the more

¹⁶ *Zentralbl. f. Physiol.*, xix, p. 504, 1905.

¹⁷ *Monatsh. f. Chem.*, xxiv, p. 230, 1903.

¹⁸ *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 2243, 1910.

¹⁹ *Zeitschr. f. physiol. Chem.*, lxiv, p. 75, 1910.

²⁰ *Loc. cit.*

readily soluble laevorotatory form. This was then iodized according to the Pauly method. One determination on the snow-white product gave 63 per cent iodine (theoretical 65 per cent). The slightly lower value may be due to an admixture of a small amount of di-iodohistidine anhydride.

Iodized tryptophane. Tryptophane was prepared from commercial casein by the Hopkins-Cole method.²¹ Several attempts were made to iodize the pure crystals by the method of Neuberg,²² but in no case was a substance obtained containing more than 6.3 per cent iodine. The preparation finally made for physiological testing was obtained by dissolving one milligram molecule of tryptophane in 4 cc. of $\frac{N}{2}$ NaOH, cooling by immersing in ice water and, while keeping cool and stirring well, adding drop by drop 6 cc. of aqueous N iodine solution. The mixture was allowed to stand at ice box temperature for twenty-four hours, then filtered off. The precipitate was well washed with cold water and dried over sulphuric acid in a vacuum desiccator. The product obtained is light brown in color, readily soluble in alkalis, reprecipitated on acidifying and liberates a very small amount of iodine to chloroform on shaking therewith. Duplicate determinations on this gave 41.5 and 41.9 per cent iodine (the theoretical for mono- and di-iodo-tryptophane are 38.4 per cent and 55.7 per cent respectively).

B. Methods.

Determination of iodine. The Hunter²³ method with slight modifications was employed. The material to be analyzed, taken in quantities of 0.05-2 grams was mixed with 15 grams of fusion mixture and covered with 10 grams of fusion mixture as suggested by Hunter. To conduct the fusion the Roger's ring burner was found to be much more satisfactory in ensuring a uniform rapid heating without overheating. With the size of the flame once determined one finds ten minutes to be ample time to give a satisfactory, easily removable fusion. In the treatment with alkaline hypochlorite it was considered best to warm to 40°C. for ten minutes. In acidifying it is very important to make sufficiently acid and then always to the same degree. Sulphuric acid of 25 per cent strength was used here and since the same amounts of fusion mixtures and hypochlorite were used in each case the acidity was well controlled by always adding the same amount of acid. In removing the excess of chlorine gentle boiling was continued for forty minutes after the negative test of the vapors by starch iodine paper. In this way the blank test on the reagents never was more than 0.1 cc. of a $\frac{N}{200}$ $Na_2S_2O_3 \cdot 5H_2O$ solution.

Physiological testing by the Hunt method. The method employed was that of feeding the same quantity of iodine, in the different combinations, to white mice in such a manner as to make as certain as possible the entire consumption of the material fed. In order to do this each mouse was first

²¹ *Journ. of Physiol.*, xxvii, p. 418, 1901.

²² *Biochem. Zeitschr.*, vi, p. 276, 1907.

²³ *This Journal*, vii, p. 321, 1910.

fed for three or four days with cracker dust made into pellets of known weight. At the close of this preliminary feeding the unconsumed material was weighed and from this the average amount eaten per day determined. For ten days following this period each mouse then received this weight of cracker dust, with the incorporated iodine-containing substance, in the form of pellets. The control mice were fed in the same way with plain cracker dust pellets. At the end of the 10-day feeding period the acetonitrile was injected subcutaneously. Each dose administered in series I, II and III was contained in 1 cc. of fluid; in series IV-IX, in 0.5 cc.; in series X, in 0.66 cc. In most cases the animals consumed the food very well. All the mice used were raised in the laboratory building on a diet of milk and crackers with occasional bits of lettuce until used for the experiment. Care was taken to compare mice of as nearly the same age as possible. In the tables below the litter number of each mouse is given. The ages of the mice of the various litters were as follows: Litter 2, 119 days; litter 3, 102 days; litter 4, 100 days; litter 5, 80 days; litters 6 and 10, 99 and 113 days respectively; litter 9, 115 days; litters 11-12, 125 and 135 days respectively; litters 13-14, 144 and 151 days respectively; litter 28, 85 days; litters 29-30, 59 and 66 days respectively; litters 31, 32 and 34, 95, 85 and 97 days respectively; litters 33-35, 101 and 89 days respectively; litters 36-37, 89 days; litter 38, 79 days; litters 56-60, 91-103 days.

C. Discussion of the physiological tests.

Thyreoglobulin. Series I shows that thyreoglobulin possesses the full activity per unit of iodine when compared with the dried thyroid from which it was prepared. This is also confirmed by series IV where a decomposition product obtained from the globulin still shows the complete activity per unit of iodine. The whole of the physiological activity of the gland is therefore quantitatively in the thyreoglobulin.

Metaprotein. As stated above, this still shows the full activity per unit of iodine although the percentage concentration of iodine has increased from 0.465 per cent in the thyreoglobulin to 1.52 per cent in the metaprotein.

Iodothyrim. None of the iodothyrim preparations tested was found to bring about a resistance to acetonitrile more than three-fourths of that produced by the thyroid-tissue fed mice. The indications are that these preparations are all about equally inactive. Iodothyrim is therefore less active per unit of iodine than the thyreoglobulin. See series III and V.

Primary albumose. This is still very active, as shown by series IV and VII; although the full activity per unit of iodine is not

shown to be present in every case tested. In this connection it may be mentioned that the results in series VI are of no value. This series VI, however is an illustration of irregular results, due in all probability to impure acetonitrile. The acetonitrile was taken from a freshly opened bottle and found to smell decidedly of hydrocyanic acid. Before using, it was shaken twice with saturated potassium carbonate solution, dehydrated with P_2O_5 , and twice distilled from fresh P_2O_5 . Finally it was redistilled and the fraction collected between 79 and 83°C. This distillate was used in series VI. For the later series this distillate was again purified in the same way three times and finally redistilled twice without the addition of P_2O_5 . Here the distillate was collected between 80.5 and 81.5°C.

Secondary albumose. This is much less active per unit of iodine than either the iodothyryn preparations or the primary proteoses. Series VII shows this, where the maximum dose resisted is only 40 per cent of the maximum dose resisted by the thyroid-tissue fed mice.

Amino-acids from the phosphotungstic acid precipitate and the phosphotungstic acid filtrate respectively. The results in series IX indicate that the former possess very little physiological activity as measured by the Hunt method. On the whole, however, the results here are very unsatisfactory as the mice did not eat the amino-acid mixtures well, there being two or more days' feeding left. The results indicate that these amino-acid fractions contain very little thyroid activity. This is better shown in series X where only one-tenth the quantity of iodine-containing substances was fed. Although the mice fed with dried thyroid tissue resisted an amount over two and a half times that of the control mice, still the mice fed with the same amount of iodine, but in the form of amino-acids, resisted very little, if any, more of the acetonitrile than the control mice. In other words, these amino-acid fractions show a very slight physiological activity, if indeed they possess any activity whatever.

Tetra-iodohistidine anhydride and iodotryptophane. These substances when fed in amounts representing ten times the amount of iodine fed as thyroid tissue do not appreciably increase the resistance to acetonitrile. See series II and VIII.

Table II gives a summary of the results above. The relative physiological activity is expressed (on the basis of feeding the same amount of iodine in each case) as follows: representing in each case, by 100, the largest dose of acetonitrile from which the thyroid-tissue fed mice recovered, then the other figures represent the proportions the limiting doses of the otherwise fed mice bear thereto.

TABLE II.

	RELATIVE ACTIVITY	IODINE IN THE SUBSTANCE	TOTAL IODINE IN THE TISSUE
		<i>per cent</i>	<i>per cent</i>
Thyroid tissue.....	100	0.247	100.0
Thyreoglobulin.....	100	0.465	100.0
Metaprotein.....	100	1.520	13.2
Iodothyrim.....	50-75	4.46-7.51	18.3
Primary albumose.....	80-100	0.220	3.5
Secondary albumose.....	40	0.0695	1.5
Amino-acids precipitated by phosphotungstic acid.....	0(+?)	0.0043	
Amino-acids not precipitated by phosphotungstic acid.....	0(+?)	0.0044	
Tetra-iodohistidine anhydride.....	0	65.00	
Iodotryptophane.....	0	41.70	

These results show that both the thyroid activity and iodine may be concentrated from thyroid tissue in the thyreoglobulin as well as in the metaprotein and iodothyrim from the latter. Per unit of iodine, however, we have full activity retained in the thyreoglobulin and metaprotein only. In the primary albumose fraction we have a lowering in the percentage concentration of iodine and also a slight lowering in the physiological activity per unit of iodine. In the secondary albumose this is still more marked. In the amino-acid fractions the activity is extremely low if present. In view of the researches of Hunt and Seidell with various iodine compounds and in view of the results obtained here, we cannot attribute the protective action in any of these cases to iodine itself, but to a specific iodine-containing complex in the thyreoglobulin. It is significant to note that the highest physiological activity per unit of iodine is found in the original protein and in the more complex products of hydrolysis. Since the lowest products of hydrolysis are still less active per unit of iodine than the secondary albu-

mose it indicates either that the iodine group is altered in the hydrolysis, or that the iodine-containing group when in simpler combination or when separated, does not possess the full specific thyroid activity. That the iodine-containing group when once separated would not possess the full activity is not at all unlikely, but we would be inclined to expect it to show some activity; at least when given in amounts such as were employed by Strouse and Voegtlin with iodotyrosine and by the author in the experiments with tetra-iodohistidine anhydride and iodotryptophane. The indications as to the presence of tyrosine and tryptophane in iodothyron are very favorable, both from the chemical studies on iodothyron and also from similar studies on iodine-free melanoidins.²⁴ It is not likely that the iodine is split off and then later added to the melanoidin fraction; it is more likely that it is already present in the globulin in the melanoidin-forming groups and remains in the original position in these groups, but that the groups themselves are changed in regard to each other and thus the activity affected to some extent; a poly-iodo derivative may be changed to a mono-iodo derivative and then may show decided differences in physiological activities. If this were not the case we would expect artificially iodized melanoidins to show a decided thyroid activity. Furthermore, it is not likely that sufficient hydriodic acid is split off in the early stages of the hydrolysis to yield as much iodine as is contained in the melanoidin fraction. Finally, it is not at all improbable that we here have to do with a specific iodophore group just as in hemoglobin we have the chromophore group containing the iron. The negative results with artificially iodized proteins speak strongly in favor of this view.

CONCLUSIONS.

1. The full activity of thyroid tissue is contained in the thyreoglobulin fraction when this activity is measured by the Hunt method.
2. The full activity per iodine unit is still present in the metaprotein fraction from this globulin, although the iodine content in the metaprotein fraction has been increased over threefold that of the globulin itself.

²⁴ Samuely: *Hofmeister's Beiträge*, ii, p. 355, 1902.

3. The other products of the hydrolysis studied, primary albumose, iodothyron and secondary albumose, show a gradual decrease in activity per unit of iodine in the order given.

4. The amino-acid fractions precipitated and not precipitated by phosphotungstic acid from the partially hydrolyzed thyroglobulin still contain very small amounts of iodine and per unit of iodine are either extremely low in activity or entirely inactive.

5. Tetra-iodohistidine anhydride and iodotryptophane do not possess thyroid activity as determined by the Hunt method.

I wish to express my thanks to Prof. A. P. Mathews for suggestions made in the course of the work.

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SERIES I. February 25-March 8.

MOUSE	LITTER NO.	FED DAILY WITH CRACKER DUST PLUS	FATAL DOSE OF ACETO-NITRILE	DEATH OCCURRED AFTER	DOSE OF ACETO-NITRILE FROM WHICH RECOVERY OCCURRED
			mg. per gm.	hrs.	mg. per gm.
(a) ♂.....	4		0.4	1½	
(b) ♀.....	4				0.35
(c) ♀.....	4		Gravid, not injected Escaped from cage		
(d) ♀.....	4				
(e) ♀.....	4	1 mg. dried hog thy- roid (=0.00247 mg. I)	4.49	3½	
(f) ♂.....	3		died while feeding		
(g) ♂.....	3		4.0	30	
(h) ♀.....	3	0.531 mg. thyreo- globulin (=0.00247 mg. I)	4.0	1½	
(i) ♀.....	3				3.79
(j) ♀.....	3				3.50

SERIES II. April 21-May 1.

(a) ♂.....	3		0.55	4½	
(b) ♂.....	5				0.30
(c) j (Ser.I)...	3				0.40
(d) i (Ser.I)...	3				0.45
(e) ♂.....	2	1 mg. dried hog thy- roid (=0.00247 mg. I)	5.0	3½	
(f) ♂.....	2		4.0	4	
(g) ♂.....	2		4.5	23	
(h) ♀.....	4	0.00392 mg. tetra- iodohistidine an- hydride (=0.00247 mg. I)			0.55*
(i) ♀.....	4		0.60	<1½	
(j) ♀.....	4		0.55	8½	
(k) ♂.....	3	0.0392 mg. tetra- iodohistidine an- hydride (=0.0247 mg. I)	0.55	5	
(l) ♀.....	4		3.55	48	
(m) b (Ser.I)...	4				0.60

SERIES III. May 19-29.

(a) ♂.....	6-10		0.30	5†	
(b) ♀.....	6-10				0.45
(c) ♂.....	9		died while feeding		
(d) ♂.....	9		0.50	8	

* Slight loss in injection.

† Not well when injected.

SERIES III—Continued.

MOUSE	LITTER NO.	FED DAILY WITH CRACKER DUST PLUS	FATAL DOSE OF ACETO- NITRILE	DEATH OCCURRED AFTER	DOSE OF ACETO- NITRILE FROM WHICH RECOVERY OCCURRED
			mg. per gm.	hrs.	mg. per gm.
(e) ♂.....	9	1 mg. dried hog thy- roid (=0.00247 mg. I)	3.5	7	2.9
(f) ♀.....	6-10				
(g) ♂.....	6-10		3.2	30	
(h) ♂.....	9	0.0424 mg. iodothy- rin (a) (=0.00247 mg. I)	2.0	8	1.5
(i) ♀.....	6-10		3.0	2	
(j) ♂.....	6-10				
(k) ♂.....	9	0.0329 mg. iodothy- rin (b) (=0.00247 mg. I)	<2.5	2	
(l) ♀.....	6-10		died while feeding		
(m) ♂.....	6-10		died while feeding		
(n) ♀.....	6-10	0.0554 mg. iodothy- rin (c) (=0.00247 mg. I)	died while feeding		1.5
(o) ♀.....	6-10		2.5	8	
(p) ♀.....	6-10				

SERIES IV. June 19-29.

(a) ♂.....	11-12	1 mg. dried hog thy- roids (=0.00247 mg. I)	3.0	3	2.5
(b) ♀.....	11-12				
(c) ♂.....	11-12		died while feeding		
(d) ♀.....	11-12				
(e) ♂.....	11-12	0.163 mg. metapro- tein (A ₄) (=0.00247 mg. I)			2.0
(f) ♀.....	11-12				2.5
(g) ♀.....	11-12		3.0	3	
(h) ♀.....	11-12	1.123 mg. primary albumose (A ₅) (=0.00247 mg. I)			2.5
(i) ♀.....	11-12		died while feeding		

SERIES V. August 2-12.

(a) ♂.....	13-14	1 mg. dried hog thy- roid (=0.00247 mg. I)			2.5
(b) ♂.....	13-14		died while feeding		<3.0*
(c) ♀.....	13-14				
(d) ♀.....	13-14	0.0424 mg. iodothy- rin (a) (=0.00247 mg. I)	2.8	3	
(e) ♂.....	13-14		died while feeding		2.0
(f) ♀.....	13-14				

* Slight loss in injection.

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SERIES V—Continued.

MOUSE	LITTER NO.	FED DAILY WITH CRACKER DUST PLUS	FATAL DOSE OF ACETO-NITRILE mg. per gm.	DEATH OCCURRED AFTER hrs.	DOSE OF ACETO-NITRILE FROM WHICH RECOVERY OCCURRED mg. per gm.
(g) ♀	13-14	{ 0.0556 mg. iodothy- rin (c) (=0.00247 mg. I)	died while feeding		
(h) ♀	13-14		2.5	3½	

SERIES VI. November 24-December 4.

(a) ♀	29-30	{ 1 mg. dried hog thy- roids (=0.00247 mg. I)	2.5	< 24	
(b) ♂	29-30		3.0	1½	
(c) ♂	29-30		2.0	< 18	
(d) ♂	29-30	{ 1.123 mg. primary albumose (A ₈) (=0.00247 mg. I)	died while feeding		
(e) ♀	28		2.0	36-40	
(f) ♂	29-30				2.5
(g) ♂	28	{ 3.55 mg. secondary albumose (A ₈) (=0.00247 mg. I)	1.5	< 4	
(h) ♀	28		2.0	24-36	
(i) ♂	28		1.25	20-36	

SERIES VII. January 13-23.

(a) ♀	31-34	{ 1 mg. dried hog thy- roid (=0.00247 mg. I)	died while feeding		
(b) ♂	31-34				2.0
(c) ♀	31-34				2.5
(d) ♂	31-34	{ 1.123 mg. primary albumose (A ₈) (=0.00247 mg. I)			3.0
(e) ♂	31-34				2.5
(f) ♀	31-34				2.0
(g) ♀	31-34	{ 3.55 mg. secondary albumose (A ₈) (=0.00247 mg. I)	2.8	> 6	
(h) ♀	31-34		3.0	> 8	
(i) ♂	31-34		2.0	2½	
(j) ♂	31-34	{ 3.55 mg. secondary albumose (A ₈) (=0.00247 mg. I)	1.2	> 4	
(k) ♀	31-34		1.0	24	
(l) ♀	31-34				1.2

SERIES VIII. February 17-27.

MOUSE	LITTER NO.	FEED DAILY WITH CRACKER DUST PLUS	FATAL DOSE OF ACETO- NITRILE mg. per gm.	DEATH OCCURRED AFTER hrs.	DOSE OF ACETO- NITRILE FROM WHICH RECOVERY OCCURRED mg. per gm.
(a) ♀	33-35		0.55	< 18	0.45
(b) ♀	33-35				0.40
(c) ♀	33-35				0.35
(d) ♀	33-35				
(e) ♂	33-35	1 mg. dried hog thy- roids (=0.00247 mg. I)	4.0	2	2.0
(f) ♂	36-37		died while	feeding	
(g) ♀	36-37		3.0	6	
(h) ♀	36-37				
(i) ♂	33-35	0.0059 mg. iodotryp- tophane (=0.00247 mg. I)	0.55	> 36	
(j) ♂	33-35		1.6	2½	
(k) ♀	36-37		1.0	18	
(l) ♀	36-37		0.45	> 24	
(m) ♂	33-35	0.059 mg. iodotryp- tophane (=0.0247 mg. I)	1.0	< 3	0.5
(n) ♂	33-35				
(o) ♀	33-35		0.70	< 18	

SERIES IX. March 12-22.

(a) a Ser. VIII	33-35	1 mg. dried hog thy- roid (=0.00247 mg. I)	4.0	< 6	3.5
(b) b Ser. VIII	33-35		did not eat; not injected		
(c) c Ser. VIII	33-35				
(d) n Ser. VIII	33-35				3.0
(e) ♀	38	33.6 mg. P. T. A. Ppt. 1 (=0.00247 mg. I)	died while feeding		0.8*
(f) ♀	38		died while feeding		
(g) ♂	38				
(h) ♂	38	100 mg. P.T.A. Filt.	1.0	< 3*	
(i) ♂	38	1 (=0.0024 mg. I)	0.8	< 18*	
(j) ♂	38		0.6	< 18*	

* Two or more days feeding left. This experiment is not reliable as animals were used which had recovered in previous experiments and the differences in age were too great for such young animals.

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SERIES X.

MOUSE	LITTER NO.	FED DAILY WITH CRACKER DUST PLUS	FATAL DOSE OF ACETO-NITRILE	DEATH OCCURRED AFTER	DOSE OF ACETO-NITRILE FROM WHICH RECOVERY OCCURRED
			mg. per gm.	hrs.	mg. per gm.
(a) ♂.....	56-60		0.5	<10	
(b) ♂.....	56-60		died while feeding		
(c) ♀.....	56-60				0.4
(d) ♂.....	56-60				0.35
(e) ♂.....	56-60	0.1 mg. dried hog thyroid (=0.000247 mg. I)	1.2	3½	
(f) ♂.....	56-60				1.1
(g) ♀.....	56-60				1.0
(h) ♀.....	56-60		not injected, gravid		
(i) ♂.....	56-60	5.74 mg. P. T. A. Ppt. 2 (=0.000247 mg. I)	0.5	48*	
(j) ♂.....	56-60		1.0	< 6†	
(k) ♂.....	56-60		0.8	<16	
(l) ♀.....	56-60				0.4
(m) ♂.....	56-60	5.61 mg. P. T. A. Filt. 2 (=0.000247 mg. I)	1.0	3½	
(n) ♂.....	56-60		0.8	<12	
(o) ♂.....	56-60		0.7	24	
(p) ♂.....	56-60				0.5

* Two days' feeding left.

† About one day's feeding left.

EXPERIMENTS BEARING ON THE FUNCTIONS OF THE LIVER IN THE METABOLISM OF FATS. I.

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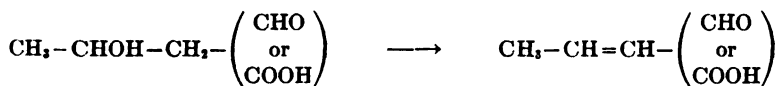
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Since the publication of Knoop's experiments on the oxidation of phenyl substituted fatty acids in the animal body, a large amount of work has been directed to the object of determining the exact nature of the intermediate reactions involved in the breakdown of fatty acids to the ultimate end products, carbon dioxide and water. It would occupy too much space to give a complete summary of this work and would, further, be needless, since an excellent account of the work has recently been published elsewhere.¹ It is sufficient to say that most of the results, so far obtained, support the hypothesis that in the catabolism of fatty acids the long chain of carbon atoms is gradually broken down by a successive series of β -oxidations involving the removal of two carbon atoms at a time. The first oxidation product is a β -ketonic acid or possibly a β -hydroxy acid, though much of the evidence points to the conclusion that β -hydroxy acids are produced by reduction of the ketonic acids. In some instances, the formation of α,β -unsaturated acids has been observed, but whether they arise by direct oxidation or indirectly, by the removal of the elements of water from a β -hydroxy acid, is not yet decided. Most of these exceedingly interesting results have been obtained by observations on the fate of phenyl or furfuryl derivatives of the lower fatty acids and it has been assumed that the same series of changes is the one which represents the mode of oxidation of the higher fatty acids when they are utilized as a source of energy in the animal body. This assumption receives support from the fact, established by Embden and his co-workers, that on perfusion of a surviving liver with blood containing caproic, octoic or decoic acids,

¹ Dakin: *Oxidations and Reductions in the Animal Body*, Longmans, Green and Company, 1912.

aceto-acetic acid was produced, this being inferred from the increased amount of acetone obtained from the blood at the end of the experiment, whereas acids containing an odd number of carbon atoms gave no aceto-acetic acid. The excretion of the "acetone bodies" in the urine in human or experimental diabetes may also be accepted as evidence in favor of the above assumption, since in these conditions it is beyond doubt that fat is being used up in relatively large quantities as a source of energy. The fact that in such conditions, and especially in the experimental ones, the liver often contains a larger amount of fat than normal, has been taken to indicate that it is to this organ that the fat first comes when it is to be oxidized, since the other active organs of the body do not exhibit this fatty infiltration to anything like the same extent. When, however, we come to examine the nature of the fat in the normal liver, it is found to contain a much larger amount of unsaturated fats than that in the fat depots or, more correctly, the fat of the liver taken as a whole is more unsaturated than the connective tissue fat. This is also true of the other active organs, such as the kidney and heart, but the difference is not quite so marked. The exact meaning of the presence of these unsaturated acids is not yet clear. Does the presence of these highly unsaturated acids indicate that they represent a stage in the normal breakdown of fat in the liver, or does it represent a stage in the synthesis of fat or are the acids essential constituents of the protoplasm of the cell which enable it to carry out functions not directly associated with the catabolism of these acids themselves?

With the knowledge at present at our disposal, it is not possible directly to answer any of these propositions, and it was with the object of trying to clear away some of the difficulties that the present work was started. We know so little about the synthesis of fats in the liver that the second possibility just mentioned cannot be put to the proof. If, however, we accept the view of Leathes, that fatty acids are synthesized by the condensation of acetaldehyde, with coincident oxidation and reduction at the appropriate stage, then it is easy to understand how unsaturated acids could arise in such a process by removal of the elements of water from the hydroxy aldehydes or acids formed, just as β -hydroxybutyric aldehyde or acid loses water and is converted into crotonic aldehyde or acid as the case may be.



Some interesting experiments on the formation and distribution of the unsaturated acids in the liver have been carried out by Leathes and his co-workers.² It has been shown that the fatty acids, contained in the lipid substances of liver, are as a rule more unsaturated than those present as glycerides, so that it seems probable that some of the unsaturated acids are destined to be built up into phosphatides and it is possible that the liver may be specially concerned in their manufacture. In a further series of experiments, in which animals (rats and cats) were fed with oils having a high iodine value, it was found on examination of the fat from the various organs that the liver fatty acids had a higher iodine value than that of the oil administered, no such change being observed in the fat from the other organs. The amount of fat, too, in the liver, appeared to be larger than normal in some cases and this also was true to some extent of the spleen. From these experiments it was concluded that "the liver takes up fat conveyed to it by the blood and changes the fatty acids in such a way as to increase their power of absorbing iodine. This may be interpreted as due either to the introduction of new unsaturated linkages, or to the transposition of existing ones from situations in which they are less liable to saturation by halogens to others in which they are more so." Another possibility may be added, namely, that the liver has some selective affinity for the highly unsaturated acids and takes them up in preference to the more saturated ones.

The work described in the present paper was undertaken with the object of determining definitely, if possible, whether saturated higher fatty acids, when taken up and metabolized in the liver, became unsaturated, as it was thought that this would tell us whether the phenomena observed by Leathes and Meyer-Wedell were due to a selective affinity of the liver for unsaturated acids. In order to accomplish this, it was necessary to have carried to the liver some saturated acid that could be separated subsequently from the acids normally present in the liver and examined with respect to its power of combining with iodine.

² Kennaway and Leathes: *Proc. Roy. Soc. Med.*, 1909; Leathes and Meyer-Wedell: *Proc. Physiol. Soc.*, 1909.

In view of Friedmann's results³ on the fate of methylated amino-acids in the organism, which showed that the methyl-amino group in these acids apparently protected them from complete oxidation, it was decided to use phenylamino-stearic acid, $C_{17}H_{31}-CH(NHC_6H_5)COOH$, in the hope that, although the α -carbon atom might be immune from attack, the long chain of carbon atoms might still be open to oxidation. Moreover, phenylamino-stearic acid is insoluble in petroleum ether and can therefore be easily separated from the fatty acids normally present in the liver. The acid, however, was not easily absorbed, so this substance had to be rejected. Further, the small amount of acid absorbed was apparently oxidized for none could be found in the liver or connective tissues. This was confirmed by examining the urine for *p*-amino-phenol. After making the urine alkaline with sodium carbonate and extracting with ether, a residue was obtained from the ether which gave a very strong indophenol reaction. Enough was not obtained for further identification. A similar result was obtained when α -phenylamino-hexanoic acid was administered. In this case 4 grams of the acid given to a cat of 3.7 kilos proved fatal. The phenylamino group in these acids is therefore not immune from attack.

The next experiments were of a different nature. Higher fatty acids substituted with bromine in the α -position were administered and an attempt was made to separate the bromo-acids from the tissue or organ fatty acids by converting the former into phenyl-amino acids—a change which is easily brought about by heating them with aniline. The free acid, however, even when mixed with meat, usually caused vomiting in cats, so this method of experiment was abandoned. In one case, in which a cat absorbed 7 grams of the acid in eighteen hours, none could be detected in the liver or connective tissues by means of aniline.

The next method chosen was the administration of cocoanut oil of which about 40 per cent of the fatty acids are volatile in steam; these acids moreover do not take up iodine. The method adopted was to separate the volatile acids by steam distillation from the liver of animals to which the oil had been administered and determine whether they had become unsaturated by estimating

³ Friedmann: *Beitr. z. chem. Physiol. u. Path.*, xi, p. 158.

the iodine absorption. Since the small amount of higher fatty acids, separated from the fatty acids of a normal liver by steam distillation, take up iodine, the method adopted had to be a differential one. Some of the experiments indicated that the acids recovered in this way from the liver had acquired the property of absorbing iodine to a slight extent. The effect was not very marked and it does not appear to be sufficiently striking to prove the assumption that the liver desaturates these acids. It is possible, however, that only the higher fatty acids, with 16, 18 or 20 carbon atoms, undergo this action to a marked extent, especially if the acids produced have to be built up into phosphatides.

The oil was administered to the animals by three methods, namely, by the mouth, by introducing soap solutions into the small intestine and by intravenous injections of fine emulsions of the oil. In the course of the experiments, some interesting results were obtained which have a bearing on the selective functions exercised by the liver in the mobilization of fat. Since cocoanut oil contains about 40 per cent of acids, volatile in steam, it was possible to estimate, roughly, the total amount of oil in the various tissues, after administration of a given amount of cocoanut oil to an animal. Many factors influence the rate at which volatile acids distil in steam so the results are only roughly quantitative. Controls have shown however that they are accurate enough to enable one to interpret them in the way that has been done.

When oil is given, mixed with food, it is not absorbed very rapidly and, after short periods of five to twelve hours, about 6 per cent of the oil absorbed is found in the liver. In the connective tissue fat its presence is just detectable. By infusion of cocoanut oil soaps into the intestine about 30 per cent of the absorbed fatty acids is found in the liver. The condition of the intestine at the end of these experiments was very abnormal, so too much stress cannot be laid upon the results. By intravenous injection of fine emulsions of the oil, none of the administered oil could be detected in the connective tissues, but from 25 to 60 per cent was found in the liver. This remarkable selective activity exhibited by the liver in the taking up of fat from the circulating blood is probably due in part to the effect of the anaesthetic. It was found, for instance, that a dog, which had cocoanut oil given by the mouth and later half a grain of morphia followed by ether anaesthesia, had

twice as much of the oil in its liver as dogs similarly fed but receiving no morphia and ether. The amount of oil in the liver was however, even then, not so great as in a dog to which the oil was given intravenously. Another factor which probably has an influence on this selective action of the liver is the rate at which the oil enters the circulation. In the feeding experiments, the oil was absorbed by cats, rarely at a greater rate than 0.5 gram per hour, whereas in the intravenous injections it was given at the rate of 0.77 to 1.37 grams per hour.

Another factor which would explain in part the divergence between the results of the feeding experiments and the intravenous injections is the possibility that the lower fatty acids in cocoanut oil, for instance, capric and lauric acids, may be absorbed, not as glycerides but as sodium salts and may not display the same tendency to accumulate in the liver as the glycerides do. It is obvious, when we consider the absorption of the sodium salts of the whole series of fatty acids, the lower members of which are absorbed without further change and the higher members largely synthesized into glycerides before entering the circulation, that there must be a gradual transition from one method of absorption to the other. It is possible then that intermediate acids, such as capric and lauric, reach the circulation partly as sodium salts, when their glycerides are introduced into the intestine; and since these acids are among those which were used to localize the cocoanut oil in the tissues because of their volatility in steam, the amount of volatile acid obtained from any organ would only indicate the *minimum* amount of cocoanut oil in that organ, because the fat entering the organ might contain a smaller percentage of volatile acids as glycerides than the original cocoanut oil. This hypothesis was tested by administering cocoanut oil to a dog and collecting the lymph from the thoracic duct during absorption of the oil. It was found that the mean molecular weight of the acids present in the lymph as glycerides was much higher than that of the acids in the oil given and was still higher than that theoretically calculated allowing for the fat normally present in lymph. The divergence in results obtained in the feeding experiments and intravenous injections may therefore be partly due to the fact that some of the lower acids from the cocoanut oil enter the circulation as sodium salts and consequently do not accumulate in the liver.

One other possibility remains to be considered, namely, that the fat taken up by the liver, when fine emulsions were introduced intravenously, was retained mechanically and the phenomenon was therefore purely one of obstruction. None of the experiments disprove this conclusively, as an emulsion has not yet been obtained so fine that a small fraction of the fat given does not remain in the lungs or spleen. This occurs with emulsions so fine that the majority of the fat particles are less than 2μ in diameter and certainly not more than 4μ . The emulsions were injected into the external jugular vein so that all the fat had to pass the pulmonary capillaries before entering the systemic circulation. In the earlier experiments, in which the emulsions were not so fine as in the later, a considerable amount of the fat remained in the lungs, the lung capillaries thus acting as filters and retaining the larger particles. Of the fat which finally entered the systemic circulation, none was found in the connective tissues, the intestinal mucous membrane or the kidneys, whereas the liver contained 30 to 40 per cent. With the finest emulsions, which resembled chyle in microscopic appearance, less than 2 per cent of the oil given was retained by the lungs and less than 3 per cent by the spleen, whereas 25 per cent was in the liver. It seems probable, therefore, that this rapid fatty infiltration of the liver by the injected cocoanut oil is a phenomenon identical with that observed in conditions of lipaemia, such as, for instance, occurs in dogs after excision of the pancreas. There seems to be every indication, therefore, when fat is brought rapidly into the circulation, that it is taken up selectively by the liver. It is hoped to make further use of this selective function, in a manner similar to that described in the present series of experiments, in order to determine the changes which occur in fatty acids other than those of cocoanut oil when taken up by the liver.

EXPERIMENTAL PART.

The estimation of total fatty acids in the liver, when carried out, was done by Liebermann's method of direct saponification, and all iodine values were determined by Wij's method.

For the separation and estimation of the volatile acids, the organ was heated with about its own weight of 50 per cent caustic potash, until complete solution was accomplished, then alcohol was

added and the heating continued until saponification of the fat was complete. The liquid was transferred to a large flask, acidified with sulphuric acid and distilled in a current of steam; 2.5 liters of distillate were collected in every case. The distillate was made alkaline with caustic potash in order to dissolve the fatty acids and then evaporated to small bulk on the water bath. This residue was transferred quantitatively to a Liebermann flask and the fatty acids soluble in petroleum ether estimated in the usual way. From a portion of the fatty acids obtained, the small amount of unsaponifiable matter usually present was removed by converting the acids into soaps in 60 per cent alcohol and extracting the solution with petroleum ether. The purified fatty acids separated from the soap solution by acidification and extraction with petroleum ether then served for the determination of the mean molecular weight.

For the volatile acids from the connective tissues, a fair sample of connective tissue from the subcutaneous tissues, the omentum and the subperitoneal fatty tissues was taken and, after saponification and liberation of the fatty acids, was distilled in steam, 2.5 liters of distillate being collected. A control, obtained by adding 1 gram of cocoanut oil to 80 grams of connective tissue fat and subsequent saponification and distillation in steam, showed that the actual weight of volatile acids obtained from the connective tissue fat gave very little quantitative indication of the amount of cocoanut oil present but the mean molecular weight of the volatile acids immediately showed that the presence of cocoanut oil in the connective tissues could be detected in this way.

For the experiments on cats, four control experiments were carried out. Three with the livers of normal cats without any addition, and one with a cat's liver to which a gram of the oil had been added before saponification. Two controls were also carried out with dog's liver on similar lines. The results are collected in table I.

It will be seen, on reference to this table, that from a normal cat's liver, on an average, 109 mgm. of volatile acids soluble in petroleum ether were obtained, with a mean molecular weight of about 250, and these absorbed 61.4 mgm. of iodine. When 1 gram of cocoanut oil was added to the liver and the subsequent treatment was the same, then the amount of volatile acids obtained

rose to 424 mgm. The iodine absorbed by these acids was 49.2 mgm. Similarly, with roughly the same amount of dog's liver, 123 mgm. of volatile acids were obtained with a molecular weight of 255.5 and absorbing 65.9 mgm. of iodine. When cocoanut oil was added to the same amount of the same liver, an increased yield of volatile acids was obtained with a corresponding decrease in their molecular weight and a slight diminution in the iodine absorption.

Feeding experiments.

In the experiment on cats, the oil was usually mixed with minced lean meat or with boiled codfish which contain less than 1 per cent of fat. When the animal was killed, the contents of the alimentary tract were taken and the fat estimated, in order to gain some idea of the amount of oil absorbed. In the experiments with dogs, the oil itself was given by the mouth and was usually readily taken. As in the experiments with cats, the stomach and intestinal contents were worked up to find the amount of oil actually absorbed. The results are collected in tables II and III.

On reference to table II, it will be seen that more volatile acids are obtained from the liver than in the control cats. In three out of the four experiments, the iodine absorption of the volatile acids is higher than in the controls, the most marked result in this direction being shown by the cat which had been receiving oil for nine days. In this experiment the iodine absorption was 50 per cent above the control average. In spite of this, the desaturation of the volatile acids can only have taken place to a slight extent, as in the most marked case, with an iodine absorption of 92 mgm., it only accounts for a rise in the iodine value of the volatile acids from 0 to 6. In all four cases the mean molecular weight of the volatile acids from the connective tissues is below the control, showing that some of the oil had been retained there. The result is most marked, as would be expected, in the cat which has received the largest amount of oil. In the other three cases, in which the animal was killed within twelve hours of administration of the oil, if allowance be made for the amount of volatile acids from a normal liver, and assuming that the amount of volatile acids obtained is equivalent to two and a half times its weight of oil, we find that between 5 and 6 per cent of the oil absorbed was in the liver.

In the experiments with dogs (table III) the presence of coconut oil in the liver is also revealed by the amount of volatile acids obtained, but it is not quite as marked as in the experiments with cats. The case in which the most notable increase in volatile acids occurred is the one in which the dog was given morphia and ether subsequent to the administration of the oil. In only three, out of the five experiments, is the iodine absorption above the average for the controls, being highest in the dog which had been anaesthetized for four and one-half hours. Three grams of urethane and ether for only half an hour appears to produce little effect on the accumulation of oil in the liver.

Infusion of soap into the intestine.

The object of these experiments was to get rapid absorption and with this the possibility of a greater accumulation of the oil in the liver. It was hoped thereby to get more satisfactory evidence as to the desaturation of the saturated volatile acids than was obtained from the feeding experiments. In these experiments, cats were used. Urethane was given, followed by ether, and cannulae were inserted into the intestine just below the pylorus and just above the ileo-caecal valve, the lower cannula being fixed to a burette. The solution to be administered was injected periodically through the upper cannula, and at the end of the experiment the intestinal contents were removed in order to determine the exact amount of fatty acid absorbed. It was found that whenever the fluid infused contained chiefly soaps, the intestine at the end of the experiment was very congested and the mucous membrane had partly desquamated so that the condition of the absorbing area had been very abnormal. On the other hand, when emulsions containing fatty acids or free hydrochloric acid were introduced, the intestine at the end of the experiment appeared quite normal and healthy. In spite of the numerous and varied mixtures introduced into the intestine, containing sometimes oil, sometimes free fatty acids or soaps, together with bile salts and glycerin, and although acid, neutral and alkaline fluids were tried, in only four of the experiments was enough absorption obtained to make it worth while searching for volatile acids in the liver. The results are collected in table IV.

The experiments show that the accumulation of the oil in the liver was much more marked than in the feeding experiments, the amount varying between 25 and 33 per cent of the fatty acid given. The iodine absorbed by the volatile acids was also higher than in the feeding experiments and still higher than in the controls. These experiments, then, indicate that desaturation of the volatile saturated acids from the oil has taken place, but, again, only to a slight extent.

Intravenous injection of emulsions.

The object of these experiments, as also that of the previous set, was to introduce fat rapidly into the circulation with the hope that more would be taken up by the liver and in consequence give a better chance for the detection of desaturation if it occurred. In all the experiments performed, the amount of fat taken up by the liver was greater than in the feeding experiments, but only slight evidence of desaturation was obtained. In the earlier experiments, the emulsions were relatively coarse, and rather less than half the oil given was retained by the lungs. These emulsions were made by shaking the oil with a 0.15 per cent emulsion of lecithin in normal saline. The emulsions usually contained about 4 per cent of oil. The amount of oil was estimated before each experiment so that the amount introduced could be accurately determined. It was discovered later that much finer emulsions could be made by using caseinogen instead of lecithin as emulsifying agent. These emulsions were prepared as follows.

Four grams of casein were heated on the water bath in a porcelain dish with 25 cc. of normal saline and 4 cc. of decinormal sodium hydroxide until solution was practically complete; 8 grams of coconut oil were now added and the mixture rubbed up with a pestle until drops of oil were no longer visible. The dish was now removed from the water bath and the rubbing up continued until with gradual cooling the mass became gelatinous. If too much saline is used to dissolve the caseinogen the mass does not set when cold and the emulsion is not quite as fine as when the liquid sets to a jelly on cooling. In case the cold mixture is still creamy, it should be warmed up again until a little water has evaporated and the grinding continued until it is cold. An alkaline salt solution was now made containing 160 cc. of normal saline and 1 cc. of decinormal soda for each gram of oil used. The gelatinous mass was rubbed up in a mortar in small portions with a few cubic centimeters of the salt solution until dissolution was complete.

Finally the creamy solution thus obtained was diluted with the remainder of the salt solution, filtered through cotton wool and finally through filter paper. If the preparation of the emulsion has been successful, the whole of the fat should be in minute particles which show Brownian movement. No particles with a diameter greater than 4μ should be present and these should be few in number. Almost the whole of the fat is usually in particles less than 2μ in diameter. On standing in the cold, practically no separation takes place.

The method of administration was as follows:

Urethane and ether were used as anaesthetics and, in the case of dogs, morphine and ether. A cannula was inserted into the external jugular vein and this was connected to the burette containing the emulsion so that the rate of entry of the fluid could be easily adjusted. The burette was surrounded by the outer tube of a Liebig's condenser through which water was allowed to circulate at a temperature of 40°C . About 100 cc. of the emulsion was usually given in a couple of hours, the rate of administration of the oil being then about 2 grams per hour. The animal was kept alive for a further two to four hours, and then killed.

The results of these experiments are collected in table V. With the exception of the last experiment, the method of determining the amount of oil in the liver was the same as in the previous experiments, that is, by distillation in steam. In the last experiment, the amount of oil going to the liver was determined by excising a lobe of the liver previous to the injection of the emulsion and then comparing the amount of fat in this lobe with the amount in the rest of the liver at the end of the experiment. The result is approximately the same as obtained by the distillation method and serves to confirm the results obtained by it. In this case the spleen was examined for the presence of cocoanut oil by comparing the mean molecular weight of the acids from a normal spleen with that of the acids found in the spleen after the injection of the oil, the mean molecular weight of the acids in the oil being 212. In this case it was found that 0.09 gram of the oil was present in the spleen. It will be seen, from the tabulated results, that there is marked evidence that the fat accumulates in the liver, but again the phenomenon of desaturation is only slightly indicated, the mean iodine absorption of the volatile acids being 71.9 mgm. as opposed to 61.4 mgm. in the controls.

In conclusion, two experiments were performed on dogs in which, two hours after a meal of cocoanut oil, an attempt to collect the

lymph flowing from the thoracic duct was made. The object of these was to determine whether the fat in the chyle had the same composition as that administered, or, if of different composition, whether the difference was caused by a failure of all the volatile acids to undergo the glyceride synthesis. In one of the experiments, although 56 cc. of lymph were collected, it contained so little fat in addition to what one might normally expect to be present in lymph, that no deductions in reference to the point in question could be made. In a second experiment only 7.5 cc. of lymph were collected as the animal died owing to a misadventure with the anaesthetic. The lymph however contained 5.37 per cent of fat and was sufficient for the purpose of the experiment. On examination of the fatty acids obtained from the chyle fat, the following results were obtained: Mean molecular weight, 236; iodine value, 19.1. The iodine value of the oil given was 7.7 and the mean molecular weight of its fatty acids, 212. Assuming the iodine value of the fat normally present in lymph to be 90, and its molecular weight 284, which are approximately what one would expect, then if enough of the normal fat were present to raise the iodine value from 7.7 to 19.1 the mean molecular weight would only be 222, whereas it was found to be 236. It thus appears as if the lower fatty acids in cocoanut oil do not completely undergo the glyceride synthesis during absorption.

SUMMARY.

1. Cocoanut oil administered to cats or dogs by the mouth can be detected in the liver in five or six hours. The amount present after times varying from five to twelve hours does not exceed 6 per cent of that absorbed.

2. If cats be anaesthetized (urethane and ether), and a solution of cocoanut oil soaps containing glycerin and bile salts be run into the small intestine, then about 30 per cent of the absorbed fatty acid is found in the liver.

3. When cocoanut oil is given to cats or dogs intravenously in the form of a very fine emulsion, containing about 4 per cent of the oil, then from 25 to 60 per cent of the oil which enters the systemic circulation is found in the liver.

4. It is probable that the greater retention of the oil by the liver, when it is administered in the form of soap or a fine emulsion, is

TABLE III.
Experiments on Dogs.

	LIVER						OBSERVATIONS
	Fatty acids	Iodine value	Molecular weight	Volatile acids from 68 gms. liver	Iodine absorbed	Molecular weight	
	<i>per cent</i>			<i>mgm.</i>	<i>mgm.</i>		
8.45 kilos, 21 gms. oil in 12 hours.....	2.65			149	77.5	235	
6.3 kilos, 3.4 gms. oil in 6 hours.....	2.76	124	288	157	62	246	
8.2 kilos, 6.7 gms. oil in 6 hours.....	2.95	118	283	182	77.2	244	
8.2 kilos, 19 gms. of oil in 7 hours.....	3.51	115.7	278	148	53.5	235	
6.5 kilos, 10.5 gms. oil in 7 hours.....	3.92	129.9	288	142	69.2	253	Ether for 1 hr. in middle of absorption period. 3 gms. urethane.
5 kilos, 12.8 gms. oil in 7 hours.....	4.57	105.3	279	212	84.7	244	0.5 gram morphia. Ether 4 hours.

TABLE IV.

COMPOSITION OF EMULSION	AMOUNT OF FATTY ACID ABSORBED	DURATION OF EXPERIMENT	LIVER				OBSERVATIONS
			Total fatty acids	Volatile acids	Iodine absorbed by volatile acids	Molecular weight of volatile acids	
	grams	hours	per cent	mgm.			
Soaps and glycerin.	2.0	4	5.70	367	78.7	218	Mucous membrane desquamated.
Soaps, glycerin, little free fatty acid.....	1.9	4	6.23	303	95.2	235	Mucous membrane desquamated.
Fatty acids, soaps, glycerin, bile salts	1.2	4.25		245	75.2	233	Mucous membrane desquamated.
Fatty acids, glycerin, bile salts.....	1.4	6.5		227	99.7		Mucous membrane normal.

TABLE Va.

ANIMAL	EMULSIFYING AGENT	OIL INJECTED	OIL RETAINED BY LUNGS	OIL ENTERING SYSTEMIC CIRCULATION	PERCENTAGE OF OIL ENTERING SYSTEMIC CIRCULATION WHICH WAS FOUND IN LIVER	DURATION OF EXPERIMENT
		grams	grams	grams		hours
Cat 1.....	Lecithin	6.0	2.57	3.43	60	4.0
Cat 2.....	Lecithin	4.65	1.56	3.09	43	5.3
Cat 3.....	Lecithin	4.35	2.13	2.22	38	5.2
Cat 4.....	Caseinogen	3.7	0.35	3.35	43	4.0
Cat 5.....	Caseinogen	3.42	0.07	3.35	25	5.5
Dog.....	Caseinogen	11.15	4.42	6.73		5.25

TABLE Vb.

ANIMAL	LIVER					KIDNEY		INTESTINAL MUCOUS MEMBRANE		CONNECTIVE TISSUE FAT	SPLEEN
	Total fatty acids	Mean molecular weight of fatty acids	Total volatile acids	Iodine absorbed by volatile acids	Mean molecular weight of volatile acids	Total volatile acids	Mean molecular weight of volatile acids	Total volatile acids	Mean molecular weight of volatile acids	Mean molecular weight of volatile acids	Total fatty acids, per cent and molecular weight
	per cent		mgm.			mgm.		mgm.			
Cat 1.....	8.56	265	932	68.0	206	244	252	81	266	251	
Cat 2.....	8.80		643	73.5	212	241	243	172	285	257	
Cat 3.....	5.83		437	85.7	218			60	246	251	
Cat 4.....	4.87		685	61.0	204						3.63% *247
Cat 5.....	4.89										
Dog.....	3.39	289	384	71.5	219					253	

*Corresponds to the presence of about 90 mgm. of coconut oil assuming normal spleen fatty acids to have a molecular weight of 284.

THE INFLUENCE OF PHLORHIZIN ON DOGS WITH ECK'S FISTULA.

BY J. E. SWEET AND A. I. RINGER.

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(Received for publication, January 30, 1913.)

The liver plays a very important rôle in the carbohydrate metabolism. Its gluco- and glycogenetic functions have been the subject of a very thorough study for over half a century, and a great deal of light has been thrown on it. Its influence on the course of pancreatic and phlorhizin glucosuria has unfortunately been limited to but three series of experiments. Markuse¹ in 1894 showed that extirpation of the pancreas in frogs does not bring about any glucosuria if the liver is extirpated at the same time. A year later Montuori² published the results of his experiments in which he showed that no glucosuria followed the extirpation of the pancreas in dogs if the blood vessels leading to the liver were tied off. In 1907 Rosenfeld³ published experiments on dogs with Eck's fistula. He showed that the injection of phlorhizin brought about no glucosuria. These experiments led their respective authors to far-reaching conclusions. Rosenfeld, in fact, went so far as to assert that no glucosuria can arise except from glucose that has passed a glycogen stage. He differentiates between "transglycogenic" and "aglycogenic" glucose. By the former, he understands glucose that has passed through the liver and has been converted into glucose. This sugar can give rise to glucosuria. By the latter, he understands glucose that has not passed the liver, and has consequently not been formed into glycogen. It is the glucose that is taken up by intravenous ad-

¹ Markuse: Über die Bedeutung der Leber für das Zustandekommen des Pankreasdiabetes, *Zeitschr. f. klin. Med.*, xxvi, p. 225, 1894.

² Montuori: Sull'importanza del fegato nel diabete pancreatico, *Gazz. d. Osped. ed. clin.*, 1895, no. 16.

³ Rosenfeld: Die Oxydationswege des Zuckers, *Berl. klin. Wochenschr.*, lii, 1907.

ministration or the glucose that circulates after the extirpation of the liver or Eck's fistula. These conclusions apparently fit in with other experiments of the author in which he showed that more glycogen arises in the liver after feeding of glucose *per os*, than after injecting intravenously. He also found that phlorhizinized animals eliminate more glucose after feeding of glucose *per os* than after subcutaneous injection. These experiments are of such great importance in the physiology of glucosuria, that a repetition of them seemed desirable.

Five dogs were operated upon. The method of operation was described by one of us (Sweet).⁴ All dogs were brought to autopsy and the existence of the fistula verified. The animals were phlorhizinized in the usual manner, and in none of the animals did the phlorhizin fail to bring about glucosuria, nor was there any deviation from the usual course. The outcome of our experiments undermines the very foundation of Rosenfeld's theory, and an analysis of his other experiments gives them an entirely different meaning. He gave one phlorhizinized dog 100 grams of glucose *per os*. Seventy-eight grams of glucose were recovered in the urine. The same dog, after it had overcome the effects of phlorhizin, received another 100 grams of glucose intravenously. This caused a temporary glucosuria with the elimination of 21 and 23 grams of glucose in two cases. The animal was then phlorhizinized as before and 100 grams of glucose given intravenously. Only 36 grams of glucose appeared in the urine, 15 grams more than normally. This led Rosenfeld to the conclusion "*dass die intravenös gegebene Glucose von diabetischen Hunden unvergleichlich besser vertragen wird als dieselbe Glucose wenn sie per os aufgenommen wird.*" He reasons that the orally-fed glucose gives rise to glycogen and hence the glucose that comes from it is not burnt readily and is thrown out in larger quantities in glucosuria, whereas the intravenously-given glucose does not yield to glycogen, and hence it is burnt.

In the light of our present understanding of phlorhizin glucosuria, it does not seem necessary to explain so simple an experiment in so speculative a way. It is very well established now that in phlorhizin glucosuria the animal does not lose the power

⁴ Sweet: The Artificial Anastomosis between the Portal Vein and the Vena Cava Inferior—Eck's Fistula, *Journ. of Exp. Med.*, vii, p. 2, 1905.

of burning glucose. The amount that may be burnt is a function of the ratio between the velocity of absorption of glucose and the velocity of its excretion by the kidneys. In other words, the amount burnt depends upon the amount that is circulating in the blood. Assuming the ratio of excretion to be constant, as was probably the case in Rosenfeld's dog, conditions for the combustion of sugar were certainly more favorable in the case of the intravenous administration, for there we have a sudden increase in the glucose concentration of the blood, which cannot possibly be removed by the kidneys at the rate at which it is supplied. Whereas in the administration of glucose *per os*, the absorption is a very slow one, small quantities of glucose enter the blood at a time, and it is swept out by the kidneys more completely. It is a very common experience that small quantities of glucose are recovered much more quantitatively than are large quantities.

From the aforesaid, there seems to be no reason for the acceptance of Rosenfeld's hypothesis.

Finally, we tested the extent to which the throwing out of the liver by Eck's fistula influences gluconeogenesis. One of our phlorhizinized dogs received 15 grams of glycocoll at one time and 5 grams at another time. Here again, no deviation from the normal could be obtained. The amount of "extra glucose" is similar to that obtained under ordinary conditions (Ringer and Lusk).⁵

Dog with Eck's fistula. Phlorhizin glucosuria.

Twelve-hour periods.

DATE 1912 APRIL	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	NH ₃ N	ACETONE AND ACETO- ACETIC ACID	β -HYDROXY BUTYRIC ACID	REMARKS
24	I	15.08	6.48	23.32	3.60	0.37			
24	II		6.96	25.10	3.61	0.45	0.14	0.89	{ 15 gm. glycocoll subcutan.
25	III	14.56	8.69	34.87	4.01	0.32	0.12	0.84	
25	IV		5.75	20.52	3.57	0.42	0.22	1.44	
26	V	14.20	5.24	16.85	3.21	0.40	0.25	1.56	{ 5 gm. glycocoll as above.
26	VI		6.61	22.89	3.46	0.44	0.21	1.33	
27	VII	14.00	5.48	16.70	3.05		0.20	1.18	

⁵ Ringer and Lusk: Über die Entstehung von Dextrose aus Aminosäuren bei Phlorhizinglykosurie, *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

SUMMARY.

Eck's fistulae were performed on five dogs. The dogs were kept from one week to two months, when phlorhizin was administered. The glucosuria that followed resembled in every detail that observed in normal dogs. This is contrary to the findings of Rosenfeld, who did not obtain any glucosuria at all.

The power of gluconeogenesis is not diminished in dogs with Eck's fistula.

THE INFLUENCE OF PHLORHIZIN ON A SPLEN-ECTOMIZED DOG.

BY J. H. AUSTIN AND A. I. RINGER.

(From the Departments of Research Medicine and Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)

(Received for publication, January 30, 1913.)

In a paper on the action of tissues and tissue juices on glucose, Levene and Meyer¹ found that the spleen possesses an activator for the enzyme which causes a condensation of the glucose molecule in muscle, liver, pancreas and lung. One of us (Austin) had several splenectomized dogs at his disposal, and we thought it of interest to see whether the absence of the spleen would in any way modify the course of the glucosuria after phlorhizin administration. The results were entirely negative, *i.e.*, the glucose elimination, the D : N ratio and the degree of acidosis resembled in every respect the course of glucosuria in normal dogs.

¹ This *Journal*, xi, p. 356, 1912.



A METHOD FOR DETERMINING THE SURFACE TENSION OF LIQUIDS FOR BIOLOGICAL PURPOSES.

By C. C. ERDMANN.

(From the Chemical Laboratory of McLean Hospital, Waverley, Mass.)

(Received for publication, January 31, 1913.)

In several papers, I. Traube¹ gives an account of interesting results obtained by means of capillary analysis. He emphasizes the importance of this method for biological work, since it enables us to observe reactions between components present in quantities so minute that gravimetric or volumetric methods utterly fail to demonstrate them. His apparatus, the stalagmometer, is simple in construction. It consists of a pipette whose outlet is formed by a short capillary terminating in an enlarged disc so as to offer a plane, polished surface for the formation of the drop. The resistance encountered by the liquid in passing the capillary adjusts the delivery of the drops so that, according to the viscosity of the liquid, about three to four seconds are required for their formation. Two marks on the pipette define a certain volume, and the number of drops contained in this volume is counted by an automatic device. Having established the drop number of the apparatus for distilled water, the surface tension relative to water of a liquid can easily be determined.

Trying to carry out the same idea in a different way, the following modification was adopted. A drop pipette for the delivery of a certain number of drops whose weight is to be considered is the essential part of the apparatus. The drop pipette, of a few cubic centimeters capacity, so as to enable determinations when small amounts only of substance are obtainable, is provided with a highly polished drop surface ground so as to form the base of a cone. The flow of the liquid is regulated by a narrow capillary (0.2-0.3 mm. in diameter) and thus, for the formation of the

¹ *Ber. d. deutsch. chem. Gesellsch.*, p. 44, 1911; *Biochem. Zeitschr.*, xxiv, p. 341, 1910.

drops, about five seconds are required. Drops delivered from this capillary were found to be uniform, so far as could be judged by their weight, provided that the drop surface was clean and the drop contained no air bubbles.

The details of the apparatus (made by Eimer & Amend of New York) may be seen in figure 1 of the accompanying sketch: *A*, polished drop-surface of about 5 mm. in diameter; *B*, a thin capillary of about $\frac{1}{8}$ mm.; *C*, a wider capillary of about 1 mm.; *D*, bulb of several cubic centimeters capacity (containing about 60 drops); *E*,

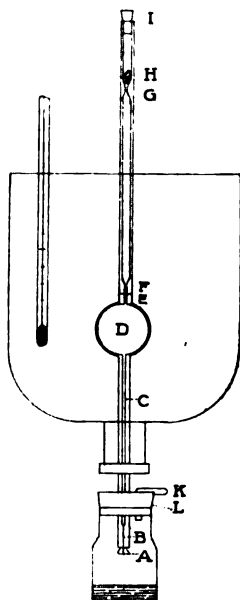


FIGURE 1.

short capillary with mark *F*; then ordinary glass tubing narrowed at *G* in order to hold in place the absorbent cotton *H* which excludes dust or particles of the stopper *I* from getting into the pipette; *K*, connection with the outside air; *L*, rubber stopper to connect the weighing bottle with the apparatus. The pipette is embedded in a water jacket for the maintenance of a uniform temperature.

The water jacket consists of an inverted Winchester bottle cut so that the neck part contains about 1.5 liters. The pipette is inserted in the neck by means of plaster of paris, and a layer of mercury protects the latter from the action of the water.

A thermometer indicates the temperature of the water jacket, and by addition of either hot or cold water a certain temperature can be maintained for some time.

Though slight differences in temperature do not materially change the size of the drops, for accurate determinations the observation of the different temperatures is important, and therefore a thermostat arrangement adjusted so as to correspond to the average room temperature may be used.

The determination of the surface tension of a liquid is carried out as follows: the clean pipette is first rinsed with the liquid by passing a small amount into the bulb and carefully drawing air through it. The short capillary will prevent the liquid from coming into contact with the absorbent cotton. Then the pipette

is filled, closed with a stopper, and about fifteen minutes are allowed for the exchange of temperature differences between the water jacket and the contents of the pipette. In the meantime the drop-surface is cleaned with fat-free filter paper. Several drops are allowed to pass, the presence of air bubbles is easily detected and eliminated and, when it is safe to assume that the protruding part of the pipette has accepted the water jacket's temperature, the pipette is closed again, and the weighing bottle attached to the apparatus by means of the rubber stopper. The weighing bottle, which has a capacity of about 50 cc., contains a layer of liquid paraffin to prevent evaporation. The withdrawal of the stopper of the pipette will start the formation of the drops, and thus 10, 20 or more drops are collected. It was found unnecessary to count the number of drops for a liquid more than once, since a certain weight corresponds to a certain number of drops.

In collecting 10 drops of the same liquid in each of two weighing bottles, the maximum difference in their weight will be found not to exceed 0.0005; the weight of a drop of water, for instance, being 0.0850 gram, this difference would amount to $\frac{1}{170}$ of a drop. Using a thermostat arrangement, in about twenty-five determinations it was found that for distilled water the maximum difference (± 0.0012) was due to the varying purity of the distilled water obtained from the laboratory still as well as to differences between the thermostat and the room temperatures.

As there is a possibility of determining the viscosity of the liquid at the same time, the use of a stop-watch was found convenient, especially for those determinations in which a larger number of drops was collected. In order to facilitate the comparison of the results obtained from different liquids, these results may be expressed as *drop numbers*, corresponding to 5, 10 or 50 grams of substance.

The following experiments were carried out by means of a drop pipette which was surrounded by a water jacket without thermostat.

A solution of serum (rabbit) was prepared, 1 : 100 in 0.85 per cent sodium chloride. Having established its drop number, to 50 cc. of serum were added 1, 2, 5 and 10 cc. of $\frac{N}{100}$ acid in the one line of experiments and the same amount of alkali in the other. After standing for about twelve hours at room temperature, the solutions were filtered (Schleicher & Schüll, 591) and 1, 2 or 3 times 10 drops were collected. The weight of 10 drops divided into 50 (grams of substance) furnished the following drop numbers.

Temperature of the water jacket = 16.2° - 16.4°.

SUBSTANCE	WEIGHTS (GRAMS) FOR 10 DROPS	DROP NO.
0.85 per cent NaCl solution.....	0.8263; 0.8266	605.0
50 cc. + 5 cc. $\frac{N}{100}$ NaOH.....	0.8253 (not filtered)	605.7
50 cc. + 5 cc. $\frac{N}{100}$ HCl.....	0.8262 (not filtered)	605.1
Serum 1 : 100.....	0.8213; 0.8212; 0.8213	606.9
From another rabbit.....	0.8214; 0.8213	608.9
The same 3 days later.....	0.8212	609.0
The same 7 days later.....	0.8051 (decomposed)	621.0
Serum + $\frac{N}{100}$ HCl		
50 cc. serum + 1 cc.....	0.8185; 0.8187; 0.8186	610.9
50 cc. serum + 2 cc.....	0.8038; 0.8038; 0.8038	622.1
50 cc. serum + 5 cc.....	0.7820; 0.7820; 0.7821	639.5
50 cc. serum + 10 cc.....	0.7511; 0.7518; 0.7517	665.5
Serum + $\frac{N}{100}$ NaOH		
50 cc. serum + 1 cc.....	0.8226; 0.8226; 0.8226	607.8
50 cc. serum + 2 cc.....	0.8212; 0.8213; 0.8212	609.0
50 cc. serum + 5 cc.....	0.8173; 0.8176; 0.8173	611.7
50 cc. serum + 10 cc.....	0.8130; 0.8127; 0.8128	615.0

The change in the surface tension of the NaCl solution produced by the addition of either NaOH or HCl is so slight that it can be ignored. While small amounts of acid cause a pronounced change in the surface tension of serum, it shows a certain tolerance for alkali.

Results obtained from cerebro-spinal fluid treated in the same way.

SUBSTANCE	DROP NO.
Water.....	584.3
Undiluted fluid.....	615.7
1 % solution cerebro-spinal fluid in 0.85 % NaCl...	606.7
Later determinations.....	607.3
Fluid decomposing.....	608.1
50 cc. of fluid 1 : 100 + $\frac{N}{100}$ HCl	
1 cc.....	607.5
5 cc.....	608.3
50 cc. of fluid 1 : 100 + $\frac{N}{100}$ NaOH	
1 cc.....	607.7
5 cc.....	607.8

The next determinations were carried out on syphilitic and non-syphilitic sera (which I owe to the kindness of Dr. James

H. Wright of the Massachusetts General Hospital) subjected to the Noguchi modification of the Wassermann reaction.

0.85 per cent NaCl solution = 639.4

NO.	DROP NUMBERS FOR 50 GRAMS OF SUBSTANCE		REACTION
	Incubated	Not Incubated	
2	646.0	645.1	Negative.
7	645.5	654.6	Strongly positive.
10	642.2	643.6	Positive.
13	646.6	644.7	Slightly positive.
14	644.8	644.8	Negative.
16	655.5	655.6	Negative.
17	644.6	644.5	Negative.
18	658.1	657.6	Strongly positive.

For the surface tension determination, the sera were diluted 1 : 125 with salt solution, except numbers 16 and 18, which were used in a more concentrated solution (about 1 : 50). The differences in surface tension before and after incubation, expressed as drop numbers for 50 grams of substance, are very slight, hardly exceeding the experimental error, and in accordance with the investigations of Bertolini² who was unable to obtain results analogous to those of Ascoli.³

For surface tension determinations of less dilute solutions, a modification of the above pipette was used (see figure 2), which, not being surrounded by a water jacket, offered the possibility of introducing the liquid directly into the bulb by means of a short side tube. This side tube is closed by a rubber stopper holding a thermometer. Instead of filtering the solutions they are centrifuged for about fifteen minutes at high speed; thus small solid particles are removed which would be liable to be retained in the small capillary. Before the collection of the drops is begun, fifteen minutes are allowed for the acceptance of room temperature; a slight

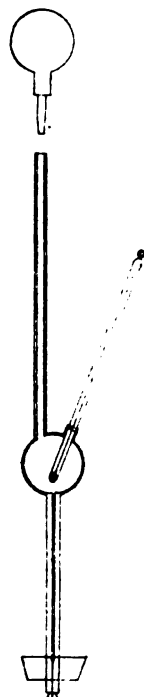


FIGURE 2.

² *Biochem. Zeitschr.*, xxviii, p. 60.

³ *Münch. med. Wochenschr.*, ii, p. 62, 1910.

pressure on the rubber bulb will start the formation of the drops. The temperature coefficient having been established, all results can be calculated for an average temperature.

The effect of heating on cow serum.

About 500 cc. of cow serum contained in a long-necked bottle were shaken in a water bath, the temperature of which was slowly raised. A thermometer immersed in the serum indicated its temperature, and from time to time (each additional 5°) a small amount was withdrawn, until coagulation rendered this impossible, and set aside for the surface tension determination. The following table indicates the results expressed in drop numbers for 50 grams of substance.

For another experiment cow serum was used to which a small amount of sodium benzoate had been added and which had been standing for three days in an ice chest.

TABLE I.
Average room temperature 20°.

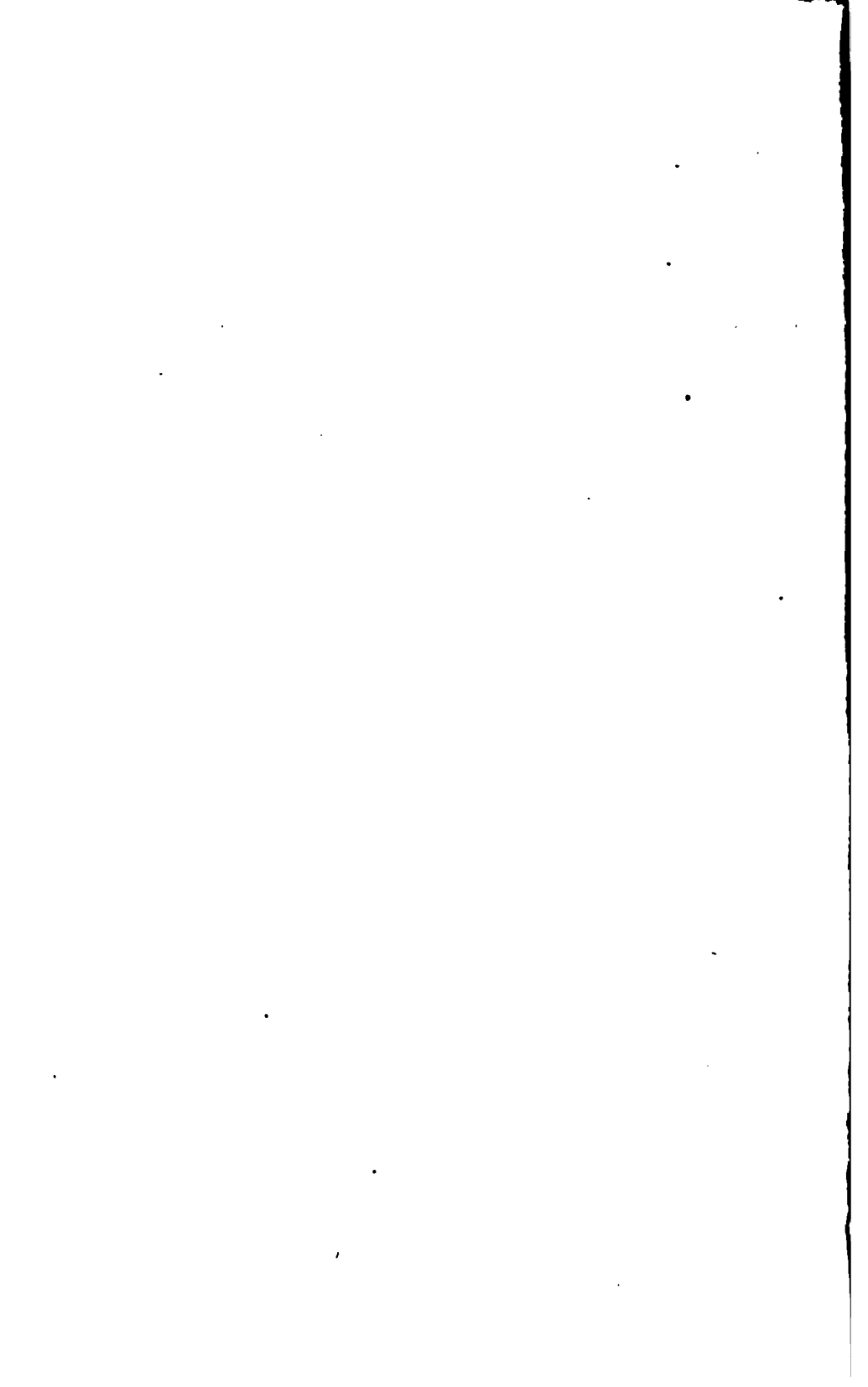
TEMPER- ATURE	WEIGHT OF 10 DROPS	DROP NO.	TEMPER- ATURE	WEIGHT OF 10 DROPS	DROP NO.
deg. C.			deg. C.		
Room	0.7688	650.7	45	0.7618	656.5
25	0.7674	651.6	50	0.7589	659.0
30	0.7636	655.0	55	0.7538	663.2
35	0.7628	655.4	60	0.7456	670.4
38	0.7629	655.4	65	0.7136	700.3
40	0.7632	655.3	70	0.6716	744.5

In both experiments the change in surface tension was found to correspond to a curve rising slowly at lower temperatures but rapidly at the beginning of coagulation, from 65°-70°. At 38°, however, a deviation occurs apparently caused by enzymes, which, at temperatures nearing that of the body, find the conditions of optimum efficiency. It was noticed that the serum, though kept in an ice chest, showed daily changes in surface tension, and therefore to a new preparation of serum about 0.1 per cent of sodium benzoate was added to prevent bacterial decomposition. Nevertheless, a similar change in surface tension occurred, and enzyme action must be held responsible for it. This would also explain

TABLE II.
Average room temperature 20°.

TEMPERATURE	WEIGHT (10 DROPS)	TIME FOR FORMATION OF 9 DROPS	DROP NO. (50 GRAMS)
<i>deg. C.</i>		<i>seconds</i>	
Room	0.7643	119	654.1
30	0.7640	115	654.5
35	0.7629	115	655.5
38	0.7633	115	655.1
40	0.7617	115	656.4
45	0.7610	115	657.0
55	0.7603	114	657.6
60	0.7547	116	662.4
65	0.7368	124	678.6
70	0.6928	219 for 4 drops	722.0

the fact that the surface tension is influenced by freezing and subsequent thawing of the serum. Changes in the viscosity of the serum are noticeable only when it has been heated nearly to coagulation temperature.



ON THE ACTION OF LEUCOCYTES ON SOME HEXOSES AND PENTOSEs.

THIRD COMMUNICATION.

CONTRIBUTION TO THE MECHANISM OF LACTIC ACID FORMATION FROM CARBOHYDRATES.

BY P. A. LEVENE AND G. M. MEYER.

(*From the Rockefeller Institute for Medical Research, New York.*)

(Received for publication, February 3, 1913.)

In previous communications the authors have demonstrated that, through the action of leucocytes, glucose is transformed into *d*-lactic acid, and that the cleavage of the sugar molecule does not proceed beyond this phase. Since then, the results obtained by us were corroborated by Embden and his co-workers, Kondo and K. v. Noorden, Jr.,¹ and by Rona and Arnheim.²

Regarding the conditions of the experiments it was stated that a certain degree of hydroxyl concentration, such as is offered by a 1 per cent Henderson phosphate mixture, was required for a successful result, and that distilled water could not be used as a medium of the reaction, since it prevented the reaction from taking place, and antiseptics, such as toluene and chloroform, acted in the same way. At the time of our first publication we had overlooked a statement of Rona and Döblin³ that the glycolysis generally produced by blood was absent when blood had been diluted with distilled water or when chloroform had been added to it.

The observations on the action of leucocytes were extended to a larger number of sugars, both hexoses and pentoses, with a view of elucidating the mechanism by which, in the organism, lactic acid is formed from sugar. It is obvious that a molecule of hexose cannot undergo a direct decomposition into two mole-

¹ *Biochem. Zeitschr.*, xlv, pp. 63 and 94, 1912.

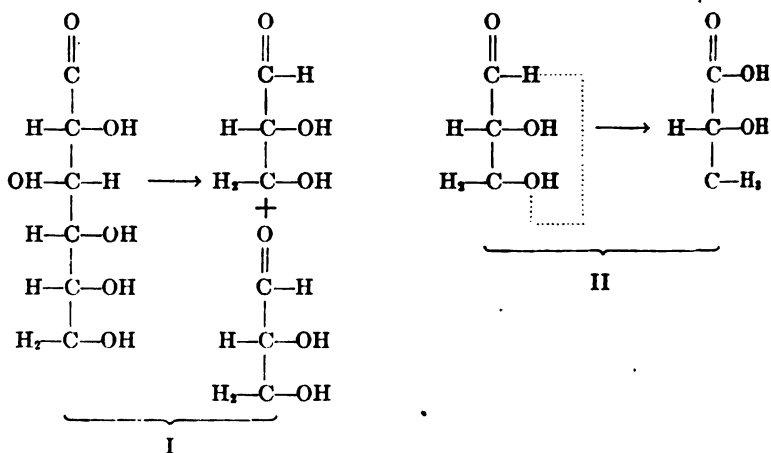
² *Ibid.*, xlviii, p. 35, 1913.

³ *Ibid.*, xxxii, pp. 489-508, 1911.

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cules of lactic acid without passing a number of intermediary transformations. In recent years there have been brought to the front a great many speculations tending to elucidate the mechanism of the reaction. They can be found in recent publications on alcoholic fermentation, lactic acid fermentation or on glycolysis.

After the publication of our previous articles on the action of leucocytes on glucose, and while the present investigation was in progress, there appeared a series of articles by Embden and his co-workers, on the basis of which Embden formulated a theory that by the action of tissue enzymes one molecule of glucose is dissociated into two molecules of glyceric aldehyde, second, that glyceric aldehyde is then transformed into lactic acid, and third, that the α -carbon atom of the glyceric aldehyde remains unaffected in course of the transformation. The mechanism may be presented in the following form.

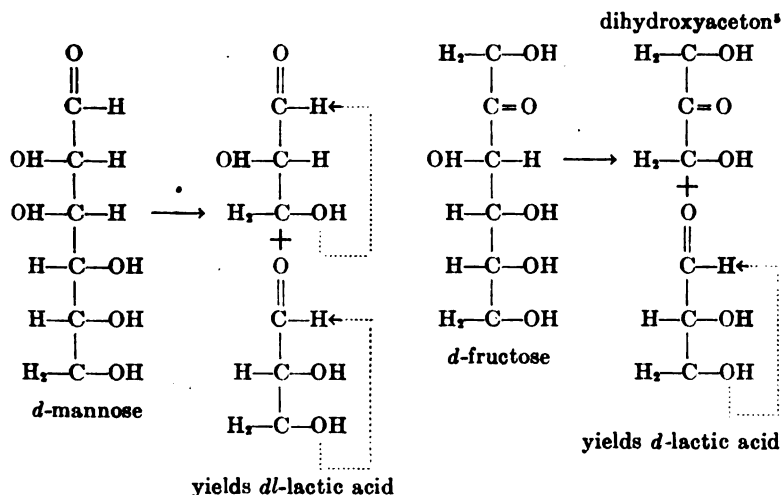


Although our own work on the mechanism of the reaction is not yet completed, yet it seems to us, that it furnishes a very definite argument against the correctness of Embden's view.⁴

It is obvious that if a molecule of glucose dissociates into two of lactic acid, according to the hypothesis of Embden, both of the latter possess the same optical character. Thus, *d*-glucose may yield two molecules of *d*-lactic acid. In this case, however,

⁴ *Biochem. Zeitschr.*, xlv, p. 108, 1912.

mannose and fructose would not be expected to yield the same result. It is evident from a glance at the configuration of the respective sugars that mannose would be expected to form *dl*-lactic acid, and fructose a mixture of *dl*- and *d*-lactic acid.



It was found in the course of our experiments that fructose, mannose and galactose all are transformed into lactic acid by means of leucocytes under the conditions reported in the previous communications. Regardless of the nature of the hexose, the lactic acid formed was invariably the *d*-form. Of course, it is important to make certain that the lactic acid obtained in the experiment did not consist of a mixture of the active and inactive forms. This possibility can be excluded in our experiments on the following grounds: first, the zinc salt of the *dl*-lactic acid is more insoluble than that of the optically active form; hence, it should be the first to crystallize if it were present in the mixture; second, the specific rotation of the zinc salt obtained from any one of the hexoses was of the same magnitude. Thus, on the basis of these experiments it does not seem possible that the formation of lactic acid is brought about by simple rearrangement in the molecule of glyceric aldehyde. Whether or not pyruvic aldehyde is the phase immediately preceding the formation of lactic acid remains to be established.

*Would be expected to yield *dl*-lactic acid.

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Attempts to bring about a dissociation of pentoses by means of leucocytes resulted negatively.

It may be mentioned here that tissues preserved under strict aseptic conditions act on sugars in a way identical to leucocytes. The results of the experiments in that connection will be communicated separately.

EXPERIMENTAL.

Leucocytes. These were obtained from dogs by injections of turpentine into the pleural cavity. The technique and further handling of the material has been described in a previous communication.

Solutions. The suspensions were made in the identical manner as the glucose experiments, in a 1 per cent Henderson phosphate solution.

Bacteriological controls. Both aerobic and anaerobic cultures were made from all leucocyte-sugar mixtures at the close of the experiment and only those analyzed which were sterile. The bacteriological examinations were made by Dr. J. Bronfenbrenner and we take this occasion to express our appreciation.

Methods of analysis. Sugar was estimated by reduction of Fehling's solution; the reduced copper was determined by Volhard's method.

Lactic acid. Since the previously reported experiments we have succeeded in obtaining a von der Heide ether extraction apparatus.⁶ The leucocyte sugar solutions were carefully made neutral to litmus, brought to a boil and the proteins coagulated with the addition of very dilute phosphoric acid. The addition of sodium sulphate crystals facilitates the precipitation of the proteins. The solution was filtered and again made neutral to litmus, and then sufficiently concentrated *in vacuo* to be contained in the ether extractor. Crystalline sodium sulphate is added to give about a one-half saturated solution and then 5 to 10 cc. of phosphoric acid are added. Extraction is allowed to proceed for at least seventy-two hours. The ether extract is then dried over anhydrous sodium sulphate and filtered. Water is added to the flask and the ether distilled off. The lactic acid in aqueous solution is converted into the zinc salt in the usual manner.

⁶ Bericht d. königl. Lehranstalt f. Weinbau in Geisenheim a. Rh., 1906, p. 23.

I. Experiments showing disappearance of sugars in mixture of sugar and leucocytes.

Mannose.

	SOLUTION USED	NEHCNS	NEHCNS PER CC.	SUGAR	LOSS	PER CENT LOSS
	cc.	cc.		per cent		
a. At beginning of experiment.....	1	20.6	20.6	6.35		
After thirty-six hours.....	1	19.8	19.8	6.10	0.25	4.10
b. At beginning of experiment.....	2	36.4	18.2	5.62		
After thirty-six hours.....	2	34.8	17.4	5.36	0.26	4.86
c. At beginning of experiment.....	1	13.6	13.6	4.19		
After thirty-six hours.....	1	12.0	12.0	3.69	0.50	11.90
d. At beginning of experiment.....	1	20.0	20.0	6.16		
After thirty-six hours.....	1	17.6	17.6	5.42	0.74	12.00
e. At beginning of experiment.....	1	19.0	19.0	5.85		
After thirty-six hours.....	1	16.3	16.3	5.03	0.82	14.00

Laevulose.

a. At beginning of experiment.....	2	37.2	18.6	6.40		
After thirty-six hours.....	2	35.4	17.7	6.09	0.31	5.10
b. At beginning of experiment.....	2	35.0	17.5	6.02		
After thirty-six hours.....	2	33.4	16.2	5.56	0.46	7.35
c. At beginning of experiment.....	1	17.6	17.6	6.05		
After thirty-six hours.....	1	16.4	16.4	5.48	0.53	8.40
d. At beginning of experiment.....	1	15.9	15.9	5.41		
After thirty-six hours.....	1	14.5	14.5	4.90	0.51	8.95

Galactose.

a. At beginning of experiment.....	2	30.8	15.4	5.61		
After thirty-six hours.....	2	29.8	14.9	5.43	0.18	3.20
b. At beginning of experiment.....	2	28.0	14.0	5.09		
After thirty-six hours.....	2	26.2	13.1	4.77	0.32	6.29

Arabinose.

a. At beginning of experiment.....	2	29.2	14.6	5.69		
After thirty-six hours.....	2	29.2	14.6	5.69	0	0
b. At beginning of experiment.....	2	28.2	14.1	5.50		
After thirty-six hours.....	2	28.4	13.2	5.53	0	0

Xylose.

a. At beginning of experiment.....	2	27.6	13.8	5.38		
After thirty-six hours.....	2	27.6	13.8	5.38	0	0

154 Lactic Acid Formation from Carbohydrates

II. Experiments showing formation of *d*-lactic acid during "Glycolysis" of mannose.

a. 150 cc. of the leucocyte mannose mixture (c, Experiment I) were extracted in a von der Heide extractor with ether. Yield of crude lactic acid = 0.2134 gram.

0.145 gram of the recrystallized salt in 2.167 grams of water in a 1 dm. tube gave a rotation, $\alpha = -0.45^\circ$.

$$[\alpha]_D^{20} = -6.7^\circ$$

b. 300 cc. of leucocyte mannose mixture (d and e, Experiment I) were together extracted with ether in a von der Heide extractor.

0.563 gram anhydrous zinc lactate was obtained. This was recrystallized and analyzed.

0.2278 gram of the recrystallized salt lost, on drying to constant weight at 110° ,

0.029 gram H_2O = 12.73 per cent H_2O .

Calculated for two molecules H_2O = 12.88 per cent.

0.0906 gram anhydrous salt after ignition

gave 0.0302 gram ZnO = 33.33 per cent ZnO .

Calculated = 33.40 per cent.

0.1344 gram zinc salt in 1.8684 grams H_2O gave a rotation in a 1 dm. tube of $\alpha = -0.47^\circ$.

$$[\alpha]_D^{20} = -7.0^\circ$$

c. 300 cc. of laevulose leucocyte mixture (c and d) were extracted with ether in a von der Heide extractor. The yield of recrystallized zinc lactate = 0.2474 gram.

0.1624 gram recrystallized salt lost, on drying to constant weight, 0.0211 gram H_2O

= 12.95 per cent H_2O .

Calculated for two molecules H_2O = 12.88 per cent.

0.1413 gram anhydrous salt was ignited and

gave 0.0469 gram ZnO = 33.20 per cent ZnO .

Calculated = 33.40 per cent.

0.1536 gram zinc salt in 2.0138 grams H_2O gave a rotation in a 1 dm. tube of $\alpha = -0.49^\circ$.

$$[\alpha]_D^{20} = -6.8^\circ$$

AN ENZYME CONCERNED WITH THE FORMATION OF HYDROXY ACIDS FROM KETONIC ALDEHYDES.

By H. D. DAKIN AND H. W. DUDLEY.

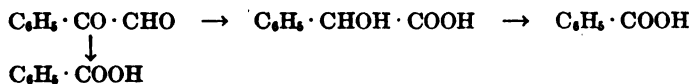
(From the Herter Laboratory, New York.)

(Received for publication, February 3, 1913.)

Since so little is known of the unstable labile substances which actively participate in the synthetic reaction of the living cell, and as it is so difficult to frame adequate experiments for the analysis of these various reactions, it seemed desirable to follow some of the changes undergone by structurally related substances which have been rendered partially stable by the incorporation of an aromatic group.

The present paper deals with the fate of phenyl glyoxal in the animal body and its decomposition by animal tissues.

Phenyl glyoxal administered to rabbits in doses of 1-1.5 grams per kilo leads to the excretion of about half a gram of optically active *l*-mandelic acid and about three-quarters of a gram of hippuric acid. No phenyl glyoxylic acid was detected. The fact that a ketonic aldehyde through enzyme action may unite with water to form an optically active hydroxy acid appears to be of some significance. The benzoic acid (hippuric acid) may originate either by the direct oxidation of the phenyl glyoxal or by the oxidation of mandelic acid or probably by both reactions.



An examination of the action of aqueous extracts of various animal tissues, including liver, pancreas, heart, skeletal muscle, kidney, blood, spleen and brain, showed that in every case an enzyme was present capable of converting phenyl glyoxal into mandelic acid and that the action of the catalyst was readily inhibited by heat. It would seem, therefore, as if the reaction was a rather general one.

The rôle that ketonic aldehydes may play in metabolism is not yet clear, but it is at least suggestive to recall that methyl glyoxal is readily obtained by the decomposition of sugar by mild hydrolysis and that by analogy methyl glyoxal should yield lactic acid, when acted upon by the enzyme previously referred to. Conversely, should the enzyme reaction prove to be reversible, as seems likely, may not this type of reaction be concerned with the actual formation of carbohydrate from lactic acid and indirectly from amino-acids? The possibility of amino-acid synthesis from methyl glyoxal must also be considered.



The testing of the above hypothesis involves somewhat difficult experiments and will take considerable time.

It is interesting to note that phenyl glyoxal readily combines with histidine, arginine, ornithine and lysine to give sparingly soluble yellow substances. These compounds are under investigation.

EXPERIMENTAL.

Three experiments were made in which phenyl glyoxal (1.5 grams) dissolved in warm water (80 cc.) was given by stomach tube to rabbits weighing about 1.5 kilos. The urine in each case was collected for about twenty-four hours and proved to be distinctly laevorotatory owing to the presence of mandelic acid. On distilling the urine, no evidence was obtained of the presence of unchanged phenyl glyoxal. The urines were acidified with phosphoric acid and extracted with ether in a continuous extractor. On concentrating the ether extract, from 1.3–1.5 grams of crystalline residue were obtained. This was dried on porous plates and the bulk of the hippuric acid obtained by direct crystallization from water. The hippuric acid melted at 185° and gave 7.7 per cent of nitrogen on analysis (calculated 7.8 per cent).

The mother liquor from the hippuric acid was shaken thrice with small portions of ether. The ether extract on evaporation gave crude mandelic acid (0.4–0.5 gram) which was readily purified by recrystallization from benzene and was completely free from hippuric acid.

In each case, some inactive mandelic acid (20 per cent) was found with the active variety, the former being probably formed by the direct hydrolysis of phenyl glyoxal other than by enzyme action. On repeated recrystallization from water the more sparingly soluble active acid, m. p. 130–132°, separates first.

0.1221 gram gave 0.2830 gram CO_2 and 0.0595 gram H_2O .

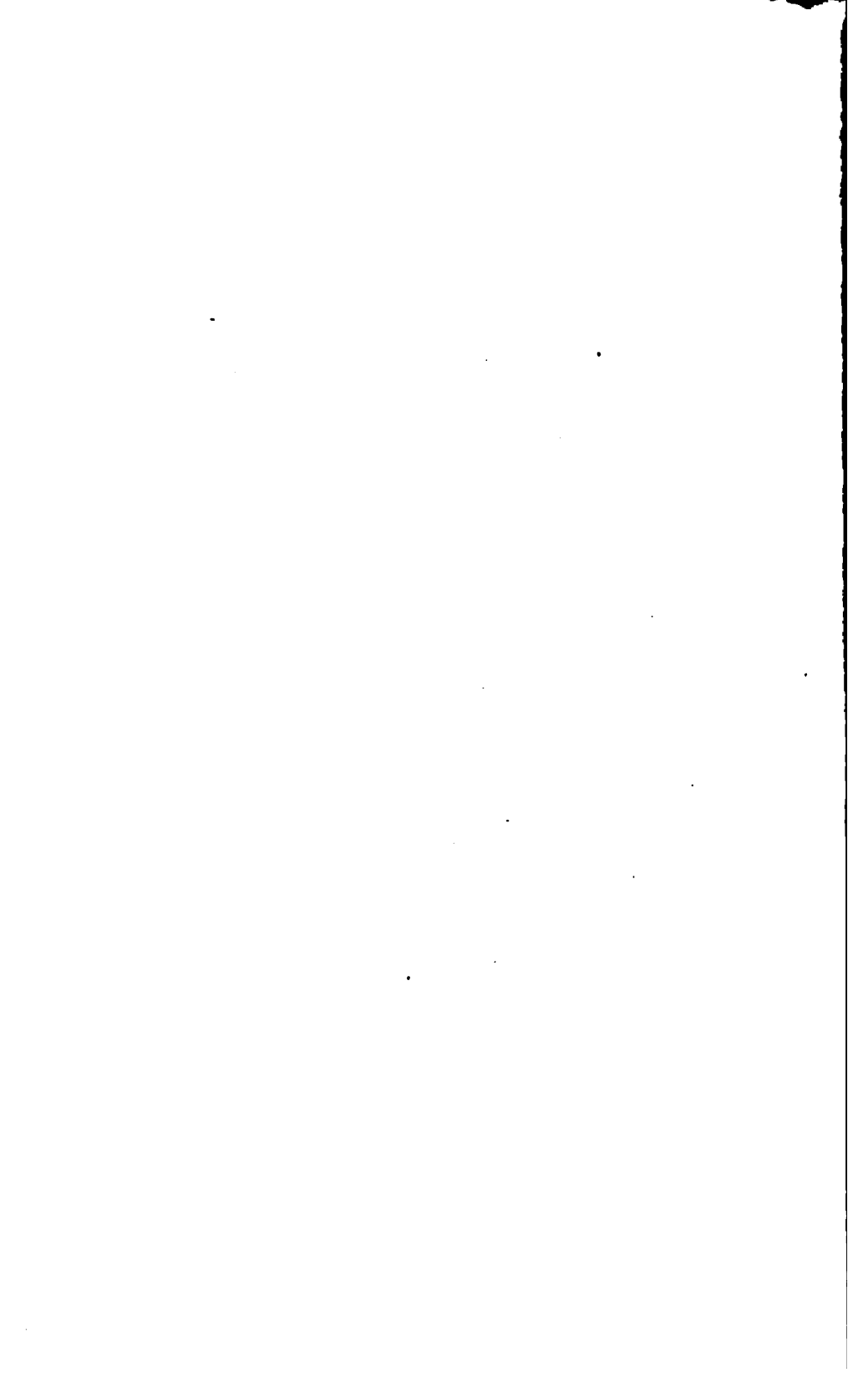
ANALYSIS.

	Found:	Calculated for $\text{C}_8\text{H}_8\text{O}_3$:
C.....	63.2	63.2
H.....	5.4	5.3

For the investigation of the action of tissues upon phenyl glyoxal, mixtures were made of from 20 to 50 grams of minced tissue with a solution containing 0.1 to 0.25 gram of phenyl glyoxal, with half its weight of sodium bicarbonate. After incubation at 37° for about twenty-four hours in the presence of toluene, the solutions were heated on a water bath with an equal volume of saturated ammonium sulphate solution containing a little phosphoric acid. The mandelic acid was extracted by shaking with ether, and the ether extract taken up in cold water (10 cc.), filtered and examined in the polarimeter in a 2 dm. tube. In every case laevo rotations of from 0.8° to 2.1°, due to *l*-mandelic acid, were observed. The mandelic acid was readily obtained in crystalline form for identification. Experiments in which the tissue extract was boiled prior to adding the phenyl glyoxal showed rotations of less than 0.18°. The results indicated that from 40 to 60 per cent of the phenyl glyoxal was converted into laevomandelic acid in every case.

A more detailed study of the action of the enzyme is in progress.

NOTE ADDED AT PROOF CORRECTION. Further investigation has shown that the enzyme above referred to is active in aqueous extracts of various tissues and that it may be roughly purified by precipitation with salts. An enzyme solution prepared from dog's liver when added to pure methyl glyoxal (4 grams), prepared according to Meisenheimer's method, effected its complete decomposition in less than ten minutes with formation of lactic acid.



**PROCEEDINGS OF THE
AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.**

SEVENTH ANNUAL MEETING.

Cleveland, Ohio, December 30, 1912-January 1, 1913.

WALDEMAR KOCH

My first duty, as President, is to pay a tribute to the memory of a worker who has fallen out of our ranks since the last meeting of our Society. Waldemar Koch was only thirty-seven years of age when he died and he was young, therefore, as the years go, but he had attained in Science a place which not rarely is the reward of later years only with others. What he did accomplish, however, was but the prologue, as it were, of a career of greater distinction which the years to come seemed abundantly to promise, and which he might hope to achieve. That hope has been denied and we who know him can but deplore our loss as well as his—ours, because he was a worthy companion enrolled with us in the scientific legion. To the older in its ranks to whom he seemed to be of the blood strain from which leaders in the legion come, as well as to the younger to whom he was a loved comrade, there remains and will remain a keen regret for his early fate, but therewith will remain also a cherished remembrance, and a thankfulness that he marched with us, even for so short a time, towards the distant watch fires of Truth.

A. B. MACALLUM.

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

PRESIDENTIAL ADDRESS.

THE ORIGIN OF MUSCULAR ENERGY: THERMODYNAMIC OR CHEMODYNAMIC?

By A. B. MACALLUM.

The question of the source and the mode of production of the energy evolved in muscular contraction is one which has been regarded, ever since the experimental method was introduced in physiology, as amongst the most fundamental and the most elusive in the whole range of vital phenomena. It was early recognized that the problem required for its solution more data than were then available or were ascertainable and that the solution itself would have to wait for those data indefinitely. There were some who held that the problem was essentially an insoluble one.

There was, however, speculation and the first to advance an explanation based on rational grounds was J. R. Mayer,¹ who in 1845 propounded the view that muscle in doing work conducts itself as a thermodynamic machine or heat engine, in which heat is produced by the combustion of material in the muscle and a portion of the heat so produced is transformed into work. This view, although it had the support of a number of isolated thinkers on the subject, was not generally received and as late as 1879 Hermann² rejected it on the score that there was not a single fact to be adduced in its support. Three years later, that is, in 1882, Fick³ formulated definitely the objections to the theory. He pointed out that if muscle is a thermodynamic engine it must work in accord with the second law of thermodynamics. Now in every thermodynamic machine, as, *e.g.*, the steam engine, the

¹ Mayer, J. R.: *Die organische Bewegung in ihren Zusammenhang mit dem Stoffwechsel*, Heilbronn, 1845.

² Hermann, L.: *Handbuch der Physiologie*, i, Pt. 1, p. 247, 1877.

³ Fick, A.: *Mechanische Arbeit*, 1882.

process by which work is produced can only be carried out by the passing of heat from one body of higher temperature A to another of lower temperature B under such conditions that a portion of the heat so transferred is converted into work. The quantity of heat so converted, that is, the amount of work done, can be ascertained accurately provided that the quantity of heat transferred is known as well as the temperatures of A and B and of the medium by which the transfer is accomplished and provided also the final state of this medium is the same as its initial state. The amount of work so produced is represented in the equation:

$$Q_o = QT_o \left(\frac{1}{T_2} - \frac{1}{T_1} \right)$$

in which Q_o indicates the quantity of heat energy converted into work, Q the amount of heat passed from the body A of higher absolute temperature, T_1 , to the body B of lower absolute temperature, T_2 , and T_o the absolute temperature of the medium. Under the most favorable circumstance $T_o = T_1$ and in the most efficient engines $Q_o = \frac{1}{4} Q$. Accordingly, in muscle, which, if a thermodynamic machine, must be considered as a highly efficient one, T_2 can only be the absolute temperature of the body or $273^\circ + 37^\circ = 310^\circ\text{C}$. and, therefore, by the equation, $T_1 = 387^\circ$ or 114°C . This involves a very much higher temperature than has been postulated to occur in muscle during contraction while the actually observed temperature after a single contraction does not exceed 0.001°C . above that of the same muscle at rest. This makes it impossible to suppose that the quantity of work resulting could be developed in this manner.

That muscle can under circumstances which are of course unusual develop energy into work thermodynamically Fick does not deny but in such cases the quantity of heat transformed into work is so small that it does not help us in believing that the transformation of heat into work occurs in ordinary muscle contraction. An instance of this he gives. A muscle, extended by a weight at 20°C . is warmed to 30°C . It shortens and lifts the weight. It does additional work if it is gradually unloaded at this temperature until the attached weight = 0. It is then cooled to 20°C . in order to allow it to resume its ordinary or unextended

length characteristic of that temperature. It is now extended by gradually increasing weight till the latter is as great as in the first instance. It is now re-warmed to 30°C. when the work done is represented by the weight raised multiplied by the distance through which it is raised. In these two cycles the muscle received heat from outside and a portion of it is transformed into work, which, however, is small in amount but its production is in accord with the second law of thermodynamics.

With the rejection of the thermodynamic hypothesis there remained two other hypotheses, one, that the energy of muscular contraction is derived from electrical energy, the other that it is derived from the energy of chemical attraction. Regarding the former little need be said. It did not attain any currency or influence speculation or stimulate experiment and, further, as a theory did not render intelligible in any respect any portion of the problem as to the origin and mode of production of the energy of muscular work.

The second of these two theories, that which postulates that muscular contraction results from the action of the attractive forces of molecules and atoms on each other had the support of Fick,⁴ Pflüger⁵ and others. This theory assumes that the molecules, on the combination of which muscular contraction depends, are arranged within the contractile substance of muscle fibre in such a way that, when the excitant action develops, they approach each other in the direction of the long axis of the muscular fibrils and thus there results a shortening of the latter. This theory is accordingly known as the chemodynamic theory. It was, until recently, practically the only resort for those who rejected the thermodynamic origin of muscular contraction but it also had its critics who claimed that it did not in the ultimate analysis afford any intelligible explanation of the phenomena of contraction. Engelmann pointed out that in a gram of muscle the elevation of temperature produced by one contraction, namely, 0.001°C. or 0.001 calorie, postulated a combustion of one-four thousandth of a milligram of carbohydrate or one-four millionth of the muscle substance. This minute portion, set in a soft

⁴ Fick A.: *Medizinische Physik*, 3te Aufl., p. 206, 1885.

⁵ Pflüger, E. F. W.: Ueber die physiologische Verbrennung in den lebendigen Organismen, *Arch. f. d. ges. Physiol.*, x, p. 329.

watery mass, must, as required by the theory, set four million parts, less one, in motion in order to bring about contraction. There is a further inherent difficulty. The molecules which generate the movement are so sparsely distributed in the muscle substance and, therefore, so far from each other that the distance between any two of them must be far greater than the distance through which molecular attraction is effective.

Engelmann⁶ accepted the thermodynamic explanation of the origin of muscular energy. He recognized and admitted that the temperature demanded in the source of the energy in the muscle to account for the amount of work done must be high, while the temperature observed is so low that it does not exceed at the most 0.005° and usually only 0.001°C. for each muscle twitch, but he explained that this is an average temperature for the whole muscle while the points where the heat is generated are infinitesimal in size. At a very minute distance from each of these points the temperature must fall so low as to be harmless to the muscle substance around each point. The muscle will no more be destroyed in this fashion than a steamship will be destroyed by heating the furnaces. "The material of combustion only will be destroyed; the vessel as a whole remains unharmed."

Engelmann advanced some facts in support of his view. He attached a moistened catgut violin string by one end, the lower, to a base and by the other to a lever which may carry a weight. Wound round the string, but at some distance from the latter, was a spiral of platinum wire whose ends were connected with the poles of a Bunsen or Grove battery of three or more cells. The base with the string and spiral was immersed in water in which at ordinary temperature the length was constant although under a weight of 25–50 grams. If a current were sent through the wire for some seconds, the water inside the spiral and about the string was heated quickly, the string immediately shortened and the lever inscribed a curve on smoked paper in many respects like the curve of the normal single twitch of a frog's muscle. In this was found what may be called a latent period, a period of quickly developing shortening followed by a period of gradually

⁶ Engelmann, Th. W.: *Ueber den Ursprung der Muskelkraft*, 2te Aufl., Leipzig, 1893: On the Nature of Muscular Contraction, *Proc. Roy. Soc.* lvii, p. 411, 1895.

decreasing relaxation. Further, the strength of the shortening power increased with the load up to a certain point. The contraction could be repeated as often as wished and the result was the same. The same phenomena were manifested by a caoutchouc string.

In these experiments the heat supplied to the string was derived from the current passing through the wire. A portion of the heat received by the string was transformed into work. Engelmann associated this power to convert heat into work with the doubly-refractive material which exists in violin strings of the type he used, in caoutchouc bands, as well as in muscle fibre and he regarded the doubly-refractive substance as directly concerned in this transformation.

These views of Engelmann were advanced in 1893 and again in 1895 and they were criticized by Fick⁷ who maintained that they were inadequate to explain the mode of production of the energy of muscular contraction. He pointed out that the violin string shortening and relaxing in the apparatus employed by Engelmann did not fulfil the conditions demanded in a reversible thermodynamic machine of the Carnot type or of that of Clausius. Also the amount of work performed by such a string is so small as to be almost negligible. Fick calculated that the transsectional area of Engelmann's apparatus was 3 cm.² and the length of the string 2 cm. It lifted 50 grams to a height of 0.5 mm. A muscle of like sectional area and like length would at least raise 10 kilos through the same distance, that is, it would do two hundred times as much work. Moreover Fick estimated that in a muscle of 1 cm.² in cross section the inotagmata, or doubly refracting elements which, according to Engelmann, transformed heat into work do not, all told, give a transsectional area of more than 0.01 mm.² and yet these delicate elements are supposed to develop all the energy of muscle contraction.

Since 1895, the thermodynamic hypothesis has undergone a transformation which is largely the result of the general acceptance of the van't Hoff-Arrhenius theory of solutions. This theory postulates that substances, electrolytes and non-electrolytes, dissolved in a fluid are in a gaseous condition and thus exercise within

⁷ Fick A.: Einige Bemerkungen zu Engelmann's Abhandlung über den Ursprung der Muskelkraft, *Arch. f. d. ges. Physiol.*, liii, p. 611, 1893.

the limits of the solution gas pressures equal to their concentrations. If now two fluids, one containing solutes and the other pure, be separated by a movable, semi-permeable membrane the latter will, according to the theory, be displaced, driven as it were by the pressure of the molecules and ions of the solute, *through* the pure fluid just as the plate of a piston in a steam cylinder is driven by the head of pressure of steam behind it. Such a displacement of the membrane postulates of course that water or the pure fluid passes through the membrane, in other words, that the solution is diluted and increased in volume. If a piston rod is connected with the membrane, work, that is, mechanical energy, may be produced. It is now possible to reverse the process by partially freezing the solution and removing therefrom the ice crystals thus formed, the water from which is added to the fluid on the other side of the membrane. The latter is then displaced in the opposite direction, work is again done and the system is once more in the initial state, that is, ready to begin the cycle of operations over again.

This is indeed the Carnot principle with but a slight variation and it is the fundamental meaning of all the views based on the principle of osmotic pressure which have been advanced to explain muscular contraction. This is, therefore, the significance of the theories advanced by McDougall,⁸ Pauli⁹ and others, which account for the contraction of the sarcostyles in muscle fibres, by the passage of water into them from without, causing them to swell and thereby to shorten. This process of "Quellung" or imbibition must be due to a force inside the sarcomeres like that which operates in the solution on one side of the semi-permeable membrane and causes the water to pass through the latter from the other side, that is, the pressure, whatever may be its causation, which obtains in the interior of a sarcostyle at the moment of excitation is greater than the pressure in the sarcoplasm. The origin of this pressure may be due to a greater number of molecules, or of

⁸ McDougall, W.: On the Structure of Cross-striated Muscle and a Suggestion as to the Nature of its Contraction, *Journ. of Anat. and Physiol.*, xxxi, p. 410, 1897; also: A Theory of Muscular Contraction, *ibid.*, xxxii, p. 187, 1898.

⁹ Pauli, Wolf.: *Kolloidchemie der Muskelkontraktion*, Dresden and Leipzig, 1912.

molecules and ions of lactic acid, for instance, or it may be due to ion-proteins, formed from anions and proteins, which cannot pass through membranes and yet exert an osmotic pressure. This is practically Pauli's explanation of the hydratising powers of colloids on which the "Quellung" or imbibition, and thereby also the contraction of muscle fibres, are based.

It is a remarkable fact that all these theories are often classified as chemodynamic, apparently because they make use of concepts and theories of physical chemistry which, it is forgotten, are largely based on thermodynamics. It is indeed generally accepted that the ultimate source in muscle fibres of the energy of contraction is to be found ultimately in certain organic compounds which on combustion yield their energy in the kinetic form and so far it may be said that the energy of muscular contraction is chemodynamic, but the energy given out as work in a gas engine is derived from the explosive combustion of the gas employed and yet we do not consider the gas engine a chemodynamic but a thermodynamic machine.

The chemodynamic theory that was accepted by Fick, Pflüger and others was based purely on the chemical affinity between oxygen on the one hand and carbon and hydrogen of organic compounds in muscle fibres on the other, and when the muscle fibre is under the influence of excitation this affinity was supposed to be allowed free play with the result that carbon and hydrogen atoms moved rapidly toward oxygen atoms or *vice versa*. As this line of movement was supposed to be in the long axis of the muscle fibre it postulated that the latter shortened. Here the primary, the fundamental, force, the sole force concerned, was supposed to be the attraction exercised on each other by the atoms of the substances involved in the combustion, and osmotic pressure was supposed to function only to the extent of promoting the diffusion into the muscle fibres of the material undergoing combustion and the removal of the waste products.

The osmotic principle if it plays the part in promoting muscular contraction required in the theories of Pauli, McDougall and others is a thermodynamic one and it is, in consequence, open to all the objections that Fick urged against thermodynamic explanations in general of muscular contraction. There are others, one of which concerns the time which explanations based on osmo-

sis demand. The process of imbibition involves the time factor. It is impossible to conceive that water will flow into the sarcostyles from the sarcoplasm, not in a third or a tenth of a second, only, but as in the case of the wing muscles of insects, in less than one-two thousandth of a second. Meigs tries to meet such a difficulty by suggesting that imbibition is not less rapid than it is in the case of a cotton string brought in contact with water for a fraction of a second. It may be pointed out that the time involved, though less, perhaps, than a second, is much greater than that postulated in the muscle fibre and that water penetrates the interstices of a string through the action of capillarity, a factor which does not play a part in muscle contraction according to the "Quellung" theories, based, as already indicated, on osmotic pressure.

Another fundamental objection to be urged against the "Quellung" theories is that the volume of each of the doubly-refracting discs of the sarcostyles in contraction is not, according to Schäfer,¹⁰ appreciably different from that of the discs in the relaxed condition and this Hürthle¹¹ confirms. McDougall, also, admits that he was unable to find an increase in volume of the sarcomeres when they contracted. Schäfer, further, points out that in many muscles, for example, those of insects and of vertebrates, the sarcoplasm between the sarcostyles is too small in quantity to account for the necessary increase of volume of the sarcomeres involved according to the theory—even if the whole of the sarcoplasm were absorbed by the fibrils. He urges, moreover, that insect wing muscle fibres contract freely in hyperisotonic solutions, that is, fluids in which the osmotic pressure greatly exceeds that which obtains in the interior of the sarcostyles.

Founded on the osmotic principle also is the theory of Zuntz,¹² advanced in 1908. This differs from the theories of McDougall and Pauli only in that it is most frankly thermodynamic. Zuntz

¹⁰ Schäfer, E. A.: On McDougall's Theory of Muscle Contraction, etc., *Quarterly Journ. of Exp. Physiol.*, iii, p. 63, 1910.

¹¹ Hürthle, K.: Ueber die Struktur der quergestreiften Muskelfasern von Hydrophilus im ruhenden und tätigen Zustand, *Arch. f. d. ges. Physiol.*, cxxvi, p. 125, 1909.

¹² Zuntz, N.: Die Kraftleistung des Thierkörpers, *Festrede zur Feier des Geburtstages Sr. Majestät des Kaisers*, etc., am 26 Januar 1908, Berlin, 1908.

estimates that, each rod in a sarcostyle having a height of 6μ and a diameter of 1μ , there would be about 5×10^{10} of such rods in a segment of muscle of 1 cm.² sectional area and 1 cm. length and these would have a surface area of 8928 cm.² available for osmotic work. Inside of these rods combustion takes place at the beginning of contraction and the carbon dioxide formed is sufficient under ordinary conditions to give an osmotic pressure of about 5 grams per 1 cm.², but as the temperature of combustion is 6400°C . this pressure is raised to 462 grams per 1 cm.² This latter exceeds the pressure in the sarcoplasm and in consequence water quickly passes from the sarcoplasm into the sarcostyles, each rod of which swells and assumes a spherical shape and, in consequence, the muscle contracts. Immediately thereafter the heat radiates from the rods into the sarcoplasm, the pressure falls and water passes out of the rods and therewith also carbon dioxide and other products formed inside the sarcostyle at the moment of contraction. The radiation of heat, the fall of pressure and the passage of water from the rods are all coincident with and cause relaxation of the sarcostyles, that is, the muscle lengthens.

There are several objections to be urged against this theory some of which have been advanced against the "Quellung" theories. These are the enormously high temperature postulated, the rate at which diffusion of water into and from the sarcostyles must take place and the impossibility of explaining how the membrane enclosing the rods should at one moment be impermeable to carbon dioxide and in less than 0.001 second thereafter become readily permeable.

A temperature of over 6000°C . in the rods of the sarcostyles is unthinkable. It would destroy the organic matter completely at the point where it would be produced and it would cause dissociation, not only of the carbon dioxide into carbon monoxide and oxygen but also of some of the water molecules into hydrogen and oxygen. Vesicles filled with carbon monoxide, hydrogen and oxygen would be formed and laceration of the internal structure of the rods would ensue.

The rate at which diffusion of water into the sarcostyles takes place is estimated by Zuntz to be extraordinarily rapid as indeed it would have to be theoretically. Assuming that oxygen diffuses

in muscle as rapidly as it does from the capillaries of the frog's lungs through the intercapillary tissue of the latter, he calculates that 0.0012 second would be required for the oxygen to pass from the sarcoplasm to the centre of a sarcofile and that, as diffusion of a substance occurs at a rate directly as the square root of its molecular weight, water would pass from the sarcoplasm into the centre of the sarcofiles in $\frac{0.0012 \times \sqrt{18}}{\sqrt{32}}$ second or about

0.0008 second. This would provide a velocity more than sufficiently great to account for a contraction which lasts only 0.03 second. According to the same calculation the carbon dioxide at the same tension would diffuse from the sarcofile into the sarcoplasm in 0.0019 second but much more rapidly than this at the tension which would obtain in the sarcofiles when the contraction develops. There would, therefore, be little or no diffusion of water into the sarcofiles if the superficial membranes of the latter were permeable to the carbon dioxide at that moment. If impermeable how do they become permeable when relaxation is to begin? Zuntz offers no explanation of this. Indeed he is silent altogether on this point.

Zuntz estimates also that the volume of the sarcofiles would in a strong contraction increase about 84 per cent. Here too may be urged the fact that McDougall, Schäfer and Hürthle have been unable to find any increase in volume of the sarcofiles in contraction.

It follows from all this review that the "Quellung" theories of muscle contraction present difficulties which are almost as great as the problem which they are intended to solve or explain. They are all based on the hypothesis that the breakdown of the compound responsible for the production of the heat of a muscle contraction immediately precedes or completely synchronizes with that contraction. This, as A. V. Hill¹³ has pointed out, is very doubtful. We have no means of demonstrating this synchronism if it obtains. Another assumption, sometimes explicitly, sometimes tacitly made, is that this heat is derived from the combustion of a carbohydrate, that is, of a glucose or glucose-holding

¹³ Hill, A. V.: The Heat Production of Surviving Amphibian Muscles during Rest, Activity and Rigor, *Journ. of Physiol.*, xlv, p. 466, 1912.

body, whereas the work of Fletcher, Hopkins and Hill seems to indicate that it is produced by the breakdown of a lactic acid complex which sets free that acid and at the same time more heat than is produced in the complete combustion of glucose. In the presence of oxygen this complex is built up again out of the lactic acid and other products of its breakdown and in that synthesis heat energy is transformed into chemical, that is, potential energy.

These observations seem to give a new aspect to the question of the origin and mode of production of muscular energy. They do not, of course, dispose finally of the "Quellung" theories. These are, as already indicated, open to serious objections on their own score alone. What is shown is that the processes involved are not so simple as postulated in the hitherto advanced thermodynamical theories of muscular contraction.

It is evident also that we cannot account for the energy of muscular contraction on the basis of a chemodynamic process such as Verworn and others held. The difficulty involved in the acceptance of such a chemodynamic theory was clearly indicated by Engelmann and the objection is irrefutable in view of the results of Fletcher's, Hopkins' and Hill's investigations. Must we then after all fall back on some thermodynamic explanation?

My answer to this is that we have still an explanation which is not a thermodynamic one and yet is not chemodynamic in the sense of that term as employed by Fick, Pflüger and Verworn. This explanation postulates as the chief factor in muscular contraction the attraction between the molecules, constituting the superficial film of a sarcostyle and forming an interface with the sarcoplasm surrounding the sarcostyle. This attraction is the cause of the surface tension in the superficial film of every fluid, semi-fluid, or semi-viscid system and obtaining, therefore, in the superficial film of each sarcostyle. In the doubly-refractive discs of insect wing muscle fibrils the tension is not equal throughout the whole of the surface film because the structure is not spherical, which it should be if the tension were uniform. This view is supported by the fact that potassium salts which are present in the doubly-refractive discs are localized or condensed at the ends of their longitudinal axes, a fact I hold due to the Gibbs-Thomson principle, according to which condensation of salts and other

solutes in a system is effected where the surface tension is lower than elsewhere. This indicates that the surface tension is lower at the ends of the discs than on their lateral or longitudinal surfaces. When the discs contract they tend to become spherical and can be seen to assume a greater curvature on their longitudinal surface, a result which would indicate that either the terminal faces develop a greater surface tension or the longitudinal surface film suffers a decrease. I am inclined to regard the latter as the correct interpretation.

How this decrease is brought about is another question. If the nerve impulse is merely a change of potential travelling along a nerve, its arrival at the surface of the fibril would diminish immediately and for a moment the attraction between the molecules on which surface tension of the superficial film depends, each sarcous disc would become more spherical and the muscle would shorten. The diminution would develop, when initiated, in less than one-tenth thousandth of a second and it would continue as long as the charge remained in the longitudinal surface of the disc.

One can, with this explanation, account for the other phenomena of contraction. The breakdown of the lactic acid precursor is one of these. It is an observed fact that chemical action at interfaces is more intense than throughout the systems forming the interfaces, especially when the tension of the latter is lowered. A momentary diminution of the surface tension on the longitudinal surface of the sarcous discs would tend to promote chemical change and this would possibly involve the breakdown of the lactic acid precursor. On the reestablishment of the surface tension in its original strength the reverse chemical action would develop, perhaps at the expense of the heat of the breakdown process or of the combustion of other constituents of the sarco-styles or sarcoplasm, or of both the sarco-styles and sarcoplasm.

The inequality of surface tension in the sarcous disc must constantly involve the expenditure of energy, energy set free in the metabolic processes which go on in the resting muscle. In the resting muscle the repair or restorative processes keep pace with those of the breakdown which are accentuated in the contracting muscle while in the relaxing the restorative predominate. It may be that the superficial molecules are formed of the lactic acid precursor.

This explanation of the origin of muscular energy has the merit of being consistent with the motor function in amoeboid movement of protoplasm, in cilia, and in the contractile stalk of *Vorticella* which is generally accepted as a surface tension effect. It falls into line with the view that in the interior as well as on the exterior of living matter evolution has been at work without a break in continuity from the simplest form to the most complex and that everything in the highest form of life is potentially in the lowest. Surface tension is the force which in the lower organisms is the sole factor in the motor function. Can we bring ourselves to regard it as a lost property in highly specialized structure like muscle fibre and accept the contractility of the latter as due to an *ad hoc* principle exemplified nowhere else than in striated muscle fibre? The free energy on the surface of living matter or of the interfaces between its different parts, is the most readily available of all the energy in such a system and the evolutionary principle must have developed out of that free energy a force which in its highest or most specialized form is as remote from the simple surface tension of a mass of primitive sarcode only as the steam engine is from the steam kettle.

It, of course, may be urged that in the final analysis the rational conception of muscle as a structure whose function is to produce mechanical work involves the thermodynamic principle. I am not inclined to deny that; for the point of view must depend, in this case, on the definition of the terms used. It can be said that the bent steel blade is a part of a thermodynamic engine because it can produce work from the energy stored up in the elastic strain but derived originally from heat energy employed in bending the blade. The high surface tension on the lateral or longitudinal surface of each sarcous element is like the energy stored in the bent steel blade and if it could be proved to be derived directly from the kinetic energy derived from metabolism in each sarcous disc the latter would be a heat engine. It would, however, be otherwise if the energy of surface tension were only remotely, not immediately, of kinetic origin. If immediately of chemical origin does the second law of thermodynamics apply here? In a Daniel cell, which may be looked upon as a chemical engine, practically all the energy liberated appears as electrical energy and as much of it as 90 per cent may be converted into

mechanical energy and could we get rid of friction theoretically the whole of the original energy liberated could be so transformed. To bring the cell back to its initial state an equivalent amount of electrical energy must be sent into the cell in the opposite direction and that amount may be produced by a heat engine turning a dynamo. The cell is, therefore, at least one degree removed from a thermodynamic machine and may be classed as a chemical engine. In that respect the sarcous disc is, I hold, also a chemical engine.

If, consequently, surface tension is the dominant factor in the production of muscular contraction muscle is not a heat engine but a chemodynamic one. The advantage of this point of view over that of the thermodynamic one lies in the fact that the theory concerned seems, in the language of the pragmatist, "to work" and it enables us to avoid labored explanations of the origin of muscular energy involving the cult of the ion and the semi-permeable membrane, or attributing thaumaturgic and inscrutable properties to colloids.

NOTE.

Attempts have been made to estimate the total surface energy available in muscle, based on a postulated value of the surface tension of protoplasm. What this latter is is not known but Bernstein assumes that it is approximately that of oil in contact with water, that is 22 dynes per centimeter. He assumes also that when muscle contracts the tension is increased in order to yield the absolute force of muscle and the energy to overcome the elasticity of muscle. What the increase must be depends on the degree of fineness of the fibrils. If each of the latter has a diameter of 19.988×10^{-5} cm. ($= 2\mu$) and the force is 3000 grams the surface tension in contraction is 326 dynes per centimeter and that of the resting fibril would be 22 dynes per centimeter. With fibrils having a diameter of 2×10^{-5} cm. ($= 0.2\mu$) the surface tension of contraction would be 36 dynes per centimeter or an increase of 14 dynes above the postulated surface tension value of resting muscle fibrils.

The firmness of the muscle fibrils is in itself an indication that their surface tension must be greater than that of oil in contact with water. Further, as Jensen has pointed out (*Anat. Hefte*, xxvii, p. 842), a thread measuring 1 mm. in diameter formed of the plasmodium of *Chondrioderma*, a Myxomycete, may, when it is in the dense condition, bear up a weight of nearly a gram. If the force engaged is surface tension it would amount to about 6000 dynes per centimeter. If the threads were more fluid there would be a decrease in this value but even at one-fiftieth dilution the tension would be 120 dynes as compared with 73 dynes for water.

In *Orbitolites*, a marine Foraminifer, the surface tension of its cytoplasm against water is 16 dynes. The cytoplasm in this form is very fluid and mobile and is, therefore, in contrast in this respect with the plasmodia of *Myxomycetes* and very possibly also with the material constituting muscle fibrils and the sarcoplasm about them.

It is not improbable, therefore, that surface tension may be very high in some forms of living matter and very low in others and, consequently, estimates of the surface energy derived from a muscle in contraction, based on the supposition that its fibrils have a surface tension as low as that of oil in contact with water, are, though interesting, far from being of assistance to us in attempting to reach a solution of the problems involved.

ABSTRACT OF SCIENTIFIC PROCEEDINGS

THE EXCRETION OF PURINE CATABOLITES IN SUNDRY TYPES OF MAMMALIA.

BY MAURICE H. GIVENS AND ANDREW HUNTER.

(*From the Department of Physiology and Biochemistry, Cornell University
Medical College, Ithaca, N. Y.*)

Allantoin has been isolated from, and shown to be a regular constituent of, the urine of the rabbit, horse, pig, cow, dog, cat, coyote, monkey, and man. To this list we can now add the opossum, porcupine, guinea-pig, sheep, and raccoon. In the case of the opossum, guinea-pig, sheep, coyote, and monkey we have collected a number of data bearing upon the extent of its daily excretion in starvation or on a purine-free diet, and upon the relation of the same to the simultaneous excretion of uric acid and purine bases. Our results in regard to the latter point are summarized in the following table, in which, for the sake of comparison, we have included (in brackets) a variety of related data from other sources.

ORDER	SPECIES	PER CENT OF TOTAL ALLANTOIN-PURINE NITROGEN		
		Allantoin	Uric acid	Purine bases
Marsupialia.....	Opossum.....	73	21	6
Rodentia.....	(Rabbit).....	94	6	
	Guinea-pig....	93	5	2
Ungulata.....	(Horse).....	79	21	
	(Pig).....	89	2	9
	Sheep.....	72	12	16
Carnivora.....	(Dog).....	97	2	1
	(Cat).....	97	3	
	Coyote.....	96	0	4
Primates.....	Monkey.....	74	0	26
	(Man).....	2	90	8

While in every case but that of man allantoin appears as the principal product of purine catabolism, it is apparent that its rôle is decidedly more prominent in some species than in others. So far as they go, the figures suggest that for each order there is a characteristic "allantoin ratio." Its values are such as to indicate that uricolytic power is greatest in carnivores, nearly as great in rodents, decidedly less in ungulates and marsupials, and practically absent in man. A strict evolutionary sequence is not apparent, although the extension of the investigation to other species and orders may reveal one. The work is being continued from this point of view.

STUDIES OF THE EXCRETION OF ACID.

By LAWRENCE J. HENDERSON AND WALTER W. PALMER.¹

(From the Chemical Laboratory, Massachusetts General Hospital.)

In experimental studies arising from the views expressed in an earlier paper by one of us,² a large number of observations upon the excretion of acid in urine have been made.

The concentration of ionized hydrogen has been estimated in about 2500 samples of urine from about 500 individuals, of whom about one-third were normal. The normal mean appears to be very nearly 1×10^{-6} N with a range of reaction from 3×10^{-8} N to 1×10^{-5} N. The mean in pathological cases is often high, and in many instances is 5×10^{-6} N, or higher, with a range from 3×10^{-8} N to 2×10^{-5} N.

We have never found a significant diminution of acidity in pathological cases, nor in any case, normal or pathological, a degree of alkalinity greater than that of blood, save after the administration of alkali.

We have confirmed the observations of Sellards³ upon the action of ingested sodium bicarbonate upon the reaction of the urine and have found a variety of cases in which large quantities of alkali were without effect upon the hydrogen ion concentration.

¹ Henry P. Walcott Fellow in Clinical Medicine, Harvard Medical School.

² L. J. Henderson: A Critical Study of the Process of Acid Excretion, *Journ. Biol. Chem.*, ix, p. 403, 1911.

³ A. W. Sellards: The Determination of Equilibrium in the Human Body between Acids and Bases with especial reference to Acidosis and Nephropathies, *Bull. Johns Hopkins Hosp.*, xxiii, p. 289, 1912.

Further, we find that such cases, when once the urine has been made alkaline, upon discontinuing alkali until the urine is once more acid, respond to alkali in a normal manner. This observation leads us to believe that Sellards's term "tolerance" is inexact, that the phenomenon is due to a drain of alkali from the body and is, in fact, a real test for the condition of acidosis. These observations extend to a much larger variety of cases, in which more accurate estimations of hydrogen ion concentration were made, than have been observed by Sellards, and were made before we were aware of his work.

We have also studied the quantity of acid excreted in the urine (excess of acid plus ammonia) in a large number of cases. The two fractions of acid excretion appear to vary independently, though they are more nearly parallel in normal cases, in which the two moieties are likely to be nearly equal.

The ammonia appears to be an index of the degree of acidosis only in those cases where β -oxybutyric acid is produced.

The relation between hydrogen ion concentration and total quantity of acid excreted appears to provide an index of the efficiency of the kidney in carrying out the important process of acid excretion; this "functional test" possesses the advantage that it involves no experimental interference.

We have reached the conclusion that mild states of acidosis are far more common than has been suspected, and that the therapeutic use of alkali in small quantities (until the urine reaches the reaction of blood) is often desirable.

None of our observations appear to afford support for the views of Martin Fischer⁴ on the cause of nephritis.

ON THE UTILIZATION OF AMMONIA NITROGEN IN THE PROTEIN METABOLISM.

By A. E. TAYLOR AND A. I. RINGER.

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)

In a series of communications by Grafe and Abderhalden in the *Zeitschrift für physiologische Chemie*, they showed that animals receiving 100 to 130 calories per kilo of body weight in the form

⁴ M. H. Fischer: *Nephritis*, New York, 1912.

of carbohydrates and fats, when given ammonium citrate, carbonate or acetate, were able to retain the nitrogen to a very considerable extent. In Grafe's experiment the animals were kept in nitrogenous equilibrium with ammonium citrate.

The experiments reported here fully confirm the findings of Grafe and Abderhalden, and also show that starving and diabetic animals may retain a considerable part of the nitrogen ingested (ammonium carbonate).

THE DETERMINATION OF ACETONE BODIES IN BLOOD AND TISSUES BY MICRO METHODS.

By W. McKIM MARRIOTT.

(From the Laboratories of Biological Chemistry of Washington University Medical School, St. Louis, Mo.)

Determinations of acetone, diacetic acid, and β -oxybutyric acid are made on from 2-5 cc. of blood drawn with a syringe directly from the veins of living animals or patients. Acetone, preformed and from diacetic acid, is distilled off and determined by the degree of turbidity it occasions in an alkaline mercury silver cyanide solution. Comparisons of turbidity are made in the nephelometer of Richards.

β -Oxybutyric acid is determined by oxidation to acetone according to the Shaffer method, after removal of blood proteins and sugar. The acetone found is determined as above.

THE COMPRESSION OF THE LUNGS BY INERT GASES.⁵

By HUGH McGUIGAN AND F. C. BECHT.

(From the Laboratory of Pharmacology of the Northwestern University Medical School.)

The compression of the lung by inert gas has been used in the past in the treatment of tuberculosis, with reported good results. This mode of treatment appears to be coming into vogue again. Our work, carried out on dogs, was undertaken primarily to determine the influence of the compression on the nutrition of the lung and secondarily, to determine the rate of absorption of the

⁵Read by title.

gas (N and H) from the pleural cavity. So far no untoward result has been found on the sound tissue. The gases are absorbed rapidly and after a few days—about five—all is gone, and the lung again fully inflated, without any apparent injury to the tissue. Repeated injections must be made if the lung is to be held in the collapsed condition. The experiments are still in progress.

**THE PHYSICO-CHEMICAL BASIS OF STRIATED MUSCLE
CONTRACTION. II. SURFACE TENSION.**

By WILLIAM N. BERG.

(*Washington, D. C.*)

In the following calculations, two quantities are calculated and then compared: (1) the energy liberated in a working muscle through increase of surface tension times diminution of area of contractile units, and (2) the external work done in lifting a weight a known distance.

Assume that in 1 cc. of muscle a right section contains 62 million rods and that there are 800 such layers, making a total of very nearly 5×10^{10} rods in 1 cc. of muscle. The lateral area of a rod diminishes from $4.8\mu^2$ when relaxed to $2.8\mu^2$ when contracted, giving a total diminution of nearly 1000 cm.^2 in 1 cc. of muscle which shortens by 0.24 cm. at the same time, i.e., $800 \times 3\mu$.

Assume the surface tension on the lateral rod surfaces to vary from that of pure acetic acid, $23 \frac{\text{dynes}}{\text{cm.}}$, when relaxed, to that of saturated sodium chloride solution, $85 \frac{\text{dynes}}{\text{cm.}}$, when contracted. Since

$$\begin{array}{ccc} \text{Surface energy liberated} & = & \text{diminution in area} \times \text{surface tension} \\ \text{(ergs)} & & \text{(cm.}^2\text{)} \quad \left(\frac{\text{dynes}}{\text{cm.}} \right) \end{array}$$

the energy liberated is, at most, 85,000 ergs. It is almost certainly less.

The energy (ergs) expended in lifting a mass of W grams through the distance D (cm.) is $W \times D \times 981$ ergs, since 1 gram (force) equals 981 dynes. Therefore in the above instance, the

85,000 ergs would lift $\frac{85,000}{0.24 \times 981} = 361$ grams, a weight that is by far too small.

It is difficult to understand how surface tension can cause isometric tetanus.

ON THE INFLUENCE OF PREGNANCY ON THE CYCLIC CHANGES IN THE UTERUS.

By LEO LOEB.

(From the Department of Pathology, Barnard Free Skin and Cancer Hospital, St. Louis.)

In previous investigations I have shown that cuts made into the uterine wall or foreign bodies introduced into the lumen of the uterus of the guinea pig about four to eight days after ovulation will lead to the formation of placentomata. I have furthermore shown that extirpation of the corpora lutea at an early stage after ovulation will prevent the formation of the placentomata. In another series of experiments I showed that extirpation of the corpora lutea within the first week after ovulation leads to a marked decrease in the length of the period of the sexual cycle in the guinea pig. The corpora lutea inhibit, therefore, the rupture of the graafian follicles. In our new investigations we examined the changes which take place in the uterine wall of the guinea pig during the different stages of the sexual period. There is a periodic change in the activity and in the morphology of the mucosa which corresponds to the various phases of the sexual cycle. At the time of ovulation, changes set in in the epithelial structures of the uterus and they are soon followed by changes in the connective tissue and blood vessels. With each new ovulation a new cycle of these changes begins. Now I found that if through early extirpation of the corpora lutea a new ovulation is accelerated simultaneously with the new ovulation a new cycle of changes sets in in the uterine wall. In order to demonstrate this fact it is necessary to ligate the fallopian tubes within the first two days after ovulation in order to prevent pregnancy from taking place. If we then examine about sixteen to twenty days after the first ovulation ovaries and uterine wall, we find new corpora lutea to have formed in the former and the cyclic changes

to have set in in the uterine wall. If, however, we ligate only one horn of the uterus, and permit pregnancy to develop in the other horn, we find upon examination of the ovaries, and of the uterine mucosa sixteen to twenty days after the previous ovulation, and after an early excision of the corpora lutea the premature ovulation in the ovaries to have taken place in a similar manner as without simultaneous pregnancy, while in the uterine wall the setting in of the new cycle has been prevented through the presence of an embryo or of a placenta in the horn of the other side. We see, therefore, that although pregnancy does not prevent the early ovulation after previous excision of the corpora lutea it prevents the setting in of a new cycle in the uterine mucosa. Pregnancy exerts, therefore, an inhibiting influence on the cyclic changes of the uterine wall, while it does not affect the early ovulation taking place after excision of the corpora lutea. Whether this inhibiting effect of pregnancy on the development of the sexual cycle is accomplished through a chemical or a nervous mechanism, or through a combination of both, cannot be decided at the present time, although it appears more probable that chemical factors play a decisive rôle in this mechanism. There is one further conclusion concerning the factors influencing the sexual cycle, to which I would like to call attention. The corpus luteum calls forth directly only certain phases in the cyclic changes in the uterine wall in the guinea pig, namely, the proliferative changes in the connective tissue of the mucosa while other changes, especially the first changes, affecting the epithelial cells of the mucosa are not directly dependent on the function of the corpus luteum. This latter conclusion is based on the observation that some of the cyclic changes in the uterine wall precede the formation of the corpus luteum.

ON THE MECHANISM OF STIMULATION BY OXYGEN WANT.

BY H. S. GASSER AND A. S. LOEVENHART.

(From the Laboratory of Pharmacology of the University of Wisconsin.)

We have demonstrated that carbon monoxide, when administered in certain dosage, causes a rise in blood pressure. We believe this to be due to stimulation of the vasomotor center. We believe the failure of other authors to observe this stimulation

to be due to the fact that too much of the gas was administered and that the second stage of the action, namely, depression was noted.

We find that the medullary centers preserve the same order in regard to their sensitiveness to hydrocyanic acid and carbon monoxide as has been shown for anaemia; that is, the respiratory center is the most sensitive, the vasomotor center is intermediate, and the cardio-inhibitory center is the least sensitive. We find that the latent period of the response of the respiratory and vasomotor centers to decreased oxidation, as brought about by means of the administration of hydrocyanic acid and carbon monoxide, is so short (on the average the respiratory center is stimulated in 4 seconds by hydrocyanic acid and in 6 seconds by carbon monoxide) that the stimulation cannot be due to abnormal metabolic products accumulated during decreased oxidation. Our conclusions may be stated as follows:

1. Oxygen want stimulates the cells of the respiratory, vasomotor, and cardio-inhibitory centers by decreasing oxidative processes within the cells of these centers.

2. Decreased oxidation does not bring about stimulation through accumulation of abnormal metabolic products.

3. We believe that decreased oxidation *per se* results in stimulation. Under conditions of decreased oxidation, it seems probable that other processes not directly requiring oxygen are increased, and that functional activity is the external expression of these latter processes.

FEEDING EXPERIMENTS RELATING TO THE NUTRITIVE VALUE OF THE PROTEINS OF MAIZE.

By THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL.

*(From the Laboratories of the Connecticut Agricultural Experiment Station
and the Sheffield Laboratory of Physiological Chemistry,
Yale University, New Haven, Connecticut.)*

According to the data now available more than one-half of the proteins of maize consists of zein, a type exhibiting such unique chemical and physical characters as to make probable that its nutritive properties differ from those of other proteins. About one-third of the proteins consists of maize glutelin, insoluble in

neutral solvents, extracted from the seed only by dilute alkalis, and yielding all the amino-acids characteristic for most other proteins. When zein forms the sole protein of the dietary, rats speedily decline in weight despite an apparently sufficient food intake. The decline is not due to digestive failure; for the food can be made adequate for maintenance over a considerable period by the addition to the food of tryptophane (which is missing among the decomposition products of zein). When half of the zein is replaced by another protein, such as casein, lactalbumin, edestin, or maize glutelin, nutritive decline can be checked. The proportion necessary varies with the different proteins. In contrast with zein, which lacks tryptophane, lysine, and glycocholl, gliadin, which is deficient in the last two only, suffices for maintenance without growth. Maize glutelin is adequate for normal growth. Foods containing equal parts of zein and maize glutelin promote nearly normal rate of growth; this applies likewise to the natural mixture of them as exhibited in so-called corn gluten. This material affords an opportunity to study the nutritive value of the two maize proteins before they have been subjected to any chemical manipulations. Animals kept on foods containing additions of both tryptophane and lysine to zein have been maintained over long periods of time. These observations all emphasize the extreme importance of tryptophane in successful dietaries. It also appears probable that the deficiency observed in the practical feeding of corn meal is explained in good part by the unique chemical constitution of zein which forms so large a part of its nitrogenous components.

**INTESTINAL OBSTRUCTION. STUDY OF A TOXIC SUBSTANCE
PRESENT IN THE INTESTINAL MUCOSA.**

By G. H. WHIPPLE.

(From the Pathological Laboratory, Johns Hopkins Medical School.)

Dogs with closed washed duodenal loops (ligatures just below pancreatic duct and just beyond duodeno-jejunal junction, with gastro-enterostomy) die in about two days with low blood pressure and temperature, vomiting and diarrhoea, the symptoms of shock seen in high obstruction and volvulus. The mucosa of these closed loops may be intact and normal except for congestion.

yet a toxic substance can be isolated from it. Normal mucosa gives no such substance. This toxic substance is active when given intravenously, intraperitoneally and subcutaneously, but is not absorbed from the normal intestine. It causes a profound fall in blood pressure and temperature, profuse vomiting and diarrhoea and collapse. Death follows in two to twenty hours with the general picture of fatal anaphylaxis. Autopsy shows a remarkable splanchnic congestion most marked in the intestinal mucosa which may be deep purple in color. Destruction of the mucosa by sodium fluoride is followed by death from peritonitis, but the distended loop contains no toxic substance, indicating that the mucosa is essential to the elaboration of this substance. Dogs injected with sublethal doses of this toxin are resistant to later injections and if a closed loop is produced in such an animal it may live six days instead of two to three days.

THE INFLUENCE OF THE PLANE OF PROTEIN INTAKE ON NITROGEN RETENTION IN THE PIG.

By E. V. McCOLLUM.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin.)

Experiments have been carried out in the following manner in order to compare the values of the nitrogen of the wheat and maize kernels, and of rolled oats for growth in the pig. The animals, weighing from 45-50 pounds, were placed in metabolism cages and fed starch during ten days, and the creatinine nitrogen output determined daily. The average amount was assumed to represent 18.5 per cent of the nitrogen from endogenous metabolism.⁶ The animals were then fed on individual grains, or with the grains plus a commercial, protein-rich by-product, so that the ration was derived in all cases from a single grain source. The nitrogen intake was adjusted at levels of 5, 7.5, 10, 15 and 20 times the endogenous requirement, in various experiments. These levels correspond approximately to 9.5, 13, 18, 28 and 37 per cent of protein in the ration. Feeding was continued about sixty days, and was followed by a ten-day period on starch. In all

⁶ McCollum: *Amer. Journ. of Physiol.*, xxix, p. 210, 1911.

cases the energy value of the rations was 100 calories per kilogram of pig per day.

The retention of nitrogen at all levels except 7.5 times the endogenous level, was in close agreement in all experiments, whether derived from wheat, oats or maize. At 5 times maintenance about 10 per cent of the ingested nitrogen was retained. At 7.5 times, the retention varied from 12-17 per cent in different experiments, due doubtless to the inferiority of certain of the pigs. At levels of 10, 15 and 20 times the endogenous requirement, the retention was 21-24 per cent of the ingested nitrogen, and was therefore independent of the source or plane of intake.

In one experiment still in progress, a pig of 43 pounds initial weight consumed 57.4 grams of nitrogen per day, derived from a mixture of wheat embryo and wheat gluten, during thirty-nine days. It is apparent that he has retained about 25 per cent of the ingested nitrogen.

Further work is in progress at the plane of 7.5 times the endogenous requirement, and at high planes of protein intake.

ENZYME SYNTHESSES.

By H. C. BRADLEY.

(From the Laboratory of Physiology of the University of Wisconsin.)

This work is an extension of that reported upon last year. A wide survey of tissues both vertebrate and invertebrate fails to show any correlation between the lipase activity of a tissue and its fatty metabolism as expressed by its ability to store up fat. In the same way there seems to be no correlation between the diastase of tissues and their glycogen content in period of nutritional abundance: certain invertebrate tissues rich in glycogen were found to exhibit but a trace or no diastatic activity. In plants a certain amount of evidence can be found in favor of the hypothesis that diastase and lipase produce the synthesis of starch and esters. It seems probable that in the plant the storage of starch in seeds and tubers is the result of diastatic synthesis, and that in some seeds and fruits the development of oils and esters is accomplished through the agency of lipase. No evidence could be found of a lactose-splitting enzyme in the active mammary glands from a number of animals. It is probable that lactase is not involved in the synthesis of lactose.

**FURTHER OBSERVATIONS ON THE INFLUENCE OF CAFFEINE ON
CREATINE AND CREATININE METABOLISM.¹**

BY W. SALANT AND J. B. RIEGER.

*(From the Laboratory of Pharmacology, Bureau of Chemistry, U. S.
Department of Agriculture, Washington, D. C.)*

The excretion of creatinine and creatine was studied in well fed and in fasting animals. In rabbits which were fed oats, a moderate increase of creatine was observed after the subcutaneous administration of doses of 100 and 150 mgs. per kilo. When food was withdrawn the amount of creatine was increased enormously in some rabbits. Creatinine was only slightly increased in the same subjects. The subcutaneous injection of 50 mgs. per kilo also increased the output of creatine in fasting rabbits as well as in animals which received oats. In some experiments, however, neither creatine nor creatinine was affected by the administration of caffeine even when food was withheld. Experiments on dogs with doses of 50–200 mgs. per kilo were negative. Creatine and creatinine metabolism was not affected in well fed or in fasting dogs by caffeine whether given by mouth or subcutaneously.

**A NEW METHOD OF DETERMINING VALENCE FROM THE
MOLECULAR COHESION.**

BY ALBERT P. MATHEWS.

(From the Laboratory of Physiology, University of Chicago.)

A new method was found of computing "*a*" of van der Waals' equation from the law of Eötvös and the formula of Thomas Young. The values thus obtained are in general similar to those obtained by the ordinary methods, but for simple substances are in general higher. If "*a*" be expressed as $N^2 M^2 K$, *N* being the number of molecules in the volume, *V*, then if the expression of $\frac{a}{V^2}$, representing molecular cohesion in van der Waals' equation, be divided in both the numerator and denominator by N^2 , we have $\frac{M^2 K}{v^2}$, the molecular cohesive pressure, since *v* is the space

¹Read by title.

at the disposal of a single molecule. The factor M I have called the mass of molecular cohesion. It was found by trial that the factor M^2K was a function of the molecular weight and the number of valences in the molecule; or $M^2K = C (\text{Mol. Wt.} \times \text{Val.})^{2/3}$, where C is a constant equal to 2.98×10^{-27} , if " a " is expressed in absolute units. This gives a means of computing valence when the critical data are known. By this method it was shown that most substances had the number of valences ordinarily ascribed to them, but that chlorine was always trivalent, argon was monovalent, nitrogen, as a gas, was monovalent and oxygen, as an element was monovalent, each molecule having two valences. By this method acetylene is shown to have ten valences; it is not, therefore, acetylidene. The trivalency of chlorine is indicated also by the optical method of determining valence and by the diamagnetic method. Various changes in graphic formulae will be necessitated by the changes of valence indicated by this method. The probable theoretical significance of this relationship of valence to cohesion is that cohesion is allied to magnetism and that the two kinds of electrons in a molecule, atomic and valence, differ in their freedom so that they can not be summed.

THE ENTRANCE OF IODINE INTO DISEASED TISSUES.

BY H. GIDEON WELLS AND O. F. HEDENBURG.

(*From the Otho S. A Sprague Memorial Institute and the University of Chicago.*)

A systematic consideration of the chemotherapy of tuberculosis rests upon an investigation of the permeability of both the tubercle bacillus and the tuberculous lesion for chemical substances of different characters. It is shown that compounds of iodine injected into tuberculous animals enter glandular tubercles with readiness, so that the proportion of iodine in such tubercles is usually greater than it is in most other tissues except the kidney; furthermore it is greater in the caseous contents than in the cellular peripheries of the tubercles. Tuberculous eyes also contain much more iodine than the normal mates. This property is shown not to depend upon any specific character of the tubercle itself, for other necrotic tissues also contain more iodine than normal tissues. The explanation offered is that normal cells are

not perfectly permeable to iodides (except perhaps kidney cells) and lose this impermeability or semipermeability when killed or injured, thus becoming entirely permeable for crystalloids present in the surrounding fluids. As the iodine content of the blood increases and decreases with absorption and elimination, so varies the iodine in the necrotic area, whether tuberculous or otherwise, indicating an absence of any chemical or physical binding of the iodine in such areas. A simple inert colloid, agar, implanted in the tissues, behaves in quite the same way.

Egg albumen injected into tuberculous pigs is found, by means of the anaphylaxis reaction, to penetrate the avascular tubercles but little if at all, even when present in the blood in large amounts. This fits with the hypothesis that the passage of iodine from the blood into the tubercles is a purely physical matter, the crystalloidal iodine compounds diffusing through the inert colloidal solution of a necrotic area practically unimpeded, while the colloidal egg albumen, according to the law of colloidal diffusion, is practically unable to diffuse through such a colloidal solution.

No evidence could be found of any tendency for iodine compounds of whatever nature to accumulate in tubercles or other necrotic areas, or to persist in such areas when disappearing from the normal tissues and the blood.

Exudates contain approximately the same proportion of iodine as the blood of the same animals, and hence any area with inflammatory edema and congestion will commonly show more iodine than normal tissues, although not usually more than the blood. No evidence was found of any specific entrance or fixation of iodine in inflammatory exudates. The iodine is distributed about alike in the fluid and solid portions of the exudate, indicating simple diffusion. Of normal tissues only the kidney seems to contain approximately as much iodine as the blood of the same animal. This may have some bearing upon its excretory function, since it indicates a greater permeability of renal cells than of other gland cells for iodides.

SARCOLACTIC ACID AND THE THEORY OF DIABETES.

BY R. T. WOODYATT.

(From the Clinical Laboratory of the Sprague Memorial Institute, Rush Medical College, Chicago.)

Theory proposed: "The function of the internal secretion of the pancreas is to dissociate glucose and perhaps other hexoses. Its action resembles that of alkali on sugars in general. It depends upon the presence in the internal secretion of an organic base, which forms a glucosate whose dissociation is high. Dissociation of sugar in the body is essential for its oxidation, reduction, polymerization, cleavage and for its participation in a great organic equilibrium which includes glycogen, glucose, split-products of glucose (*e.g.*, trioses and lactic acid), other hexoses, certain amino and fatty acids (*e.g.*, alanine, propionic acid), protein and fat. All anomalies in pancreas diabetes are explainable by lessened glucose dissociation with the changes in oxidation and polymerization and the disturbed organic equilibrium which this implies."

Lactic acid coming from glucose is, *per se*, evidence of dissociation of glucose in the body. The weights of lactic acid (as zinc lactate) found in muscles so treated as to insure maximum survival formation ran as follows:

	Grams.
Normal dogs (average of 4 determinations)	0.542
Phlorhizinized dog (D : N.=3.65 : 1; muscle-glycogen, 0) ..	0.127
Phlorhizinized dog (D : N.=2.8 : 1; muscle-glycogen, trace) ..	0.227
Phlorhizinized dog (D : N.=2.9 : 1; muscle-glycogen, 0.22) ..	0.296*
Diabetes mellitus	0.077

* Intravenous glucose injection ante mortem to produce hyperglycaemia comparable to diabetes mellitus.

THE PRESENCE OF AN ACID PRODUCING ENZYME IN *BACT. LACTIS ACIDI*.^a

BY E. G. HASTINGS AND E. B. HART.

(From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin.)

The work of Buchner, Herzog, and others has shown the presence of an enzyme in certain lactic acid-producing bacteria that are essentially different from the organisms predominating in

^a Read by title.

cheese. This intracellular enzyme, which can be demonstrated only after the disintegration of the cell, forms small quantities of lactic acid from sugar. So far as is known to us, a similar enzyme has never been demonstrated in organisms of the *Bact. lactis acidi* group. The growth of these organisms on all media is so meager that it is very difficult to obtain a sufficient amount for treatment by the methods employed by Buchner. An acid-producing enzyme in the lactic acid bacteria has, however, been demonstrated by quite different methods.

It had been noted that when a sample of raw or sterilized milk in which varying numbers of lactic bacteria had been allowed to develop, and to which a preservative, as chloroform or toluol, had been added, the cells soon disappeared or at least could no longer be detected by microscopical examination. A sample of fresh raw milk and one of the same milk heated to 97°C. for a short time were inoculated with a pure culture of *Bact. lactis acidi*. At varying periods in the development of acid, samples were removed from each and preserved with 3 per cent of toluol. In those treated soon after inoculation, a small number of bacteria were present while in those to which the preservative was added at a later stage in the development of acid, a much greater bacterial growth was present. If any enzymic action occurred, the samples should have shown a difference in the amount of acid formed corresponding to the amount of bacterial cells present.

The results seem to leave no doubt concerning the presence of an acid-forming enzyme in the organisms of the *Bact. lactis acidi* group that acts on the milk sugar. It might be thought that the increase in acidity was due to the production of amino-acids by a proteolytic enzyme. In this case the soluble nitrogen must be increased. That this does not occur has been shown by numerous investigations. The increase in acid, however, cannot be asserted to be due to the formation of lactic acid since, of course, no qualitative test could be made in the presence of the lactic acid in the milk at the beginning of the experiment.

THE INFLUENCE OF THE COMPOSITION AND AMOUNT OF THE MINERAL CONTENT OF THE RATION ON GROWTH.

By E. V. McCOLLUM AND MARGUERITE DAVIS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin.)

Young rats do not grow when fed the wheat kernel alone, or the wheat kernel plus wheat gluten to give a protein content of 18 per cent, when distilled water is given. The latter ration, with salts added to make the total content of the mineral elements closely similar to that of milk powder, has produced normal growth in rats during seventy days.

Rats grow normally during seventy-five to one hundred days on a ration consisting of pure casein, 18 per cent, dextrin, agar-agar and salt mixtures giving an inorganic content closely similar to either milk or egg yolk, and on certain other salt mixtures, in about the proportions found in milk and in eggs. With the same organic ration, fed with a salt mixture giving the ration an inorganic content closely similar to that of the wheat kernel, there is a complete suspension of growth. The addition of calcium, or the subtraction of magnesium induces growth in some degree. This is true also of rations derived from wheat and wheat gluten.

We have much experimental evidence indicating that an important relation exists between the proportion of the mineral elements in the diet, to its protein content. Normal growth has been secured during seventy days on a ration of casein, 34 per cent, dextrin, agar-agar and a salt mixture giving an inorganic content similar in composition and quantity to that of dry skim milk. With the mineral content like that of egg yolk, and fed in the same proportion as found in dry egg yolk diluted to 12 per cent protein content, considerable growth has been observed. It is noteworthy that both highly acid as well as slightly alkaline mixtures lead to good results if the composition is satisfactory.

CONNECTIVE TISSUES OF LIMULUS.

By H. C. BRADLEY.

(From the Laboratory of Physiology of the University of Wisconsin.)

Attached to the gill flaps of limulus are rods of cartilage-like tissue. Physically and histologically it resembles cartilage. Chem-

ically it is a typical sclero-protein, insoluble in water and the **ordinary** solvents, digesting readily in a tryptic mixture but **insoluble** in pepsin-HCl. It contains 14.4 per cent N; 1.6 per cent $\text{NH}_4\text{-N}$; 3.1 per cent tyrosine. On hydrolysis with strong HCl it **yields** considerable sulphate, and a small amount of a reducing **substance**. All of the ordinary protein reactions are positive. It **does** not yield sufficient gelatin to give a positive test for that **substance**.

Across the ventral portion of limulus, within the carapace, **extends** a white fibrous mass of connective tissue connecting a **large** number of muscles. Its general resemblance in appearance **and** function to mammalian tendon is further borne out by its histological appearance. Chemically it also is a sclero-protein, **free** from collagen and somewhat different from the cartilage. It contains 16.0 per cent N; no ammonia N; 2.4 per cent tyrosine; **no** oxidized sulphur, and a trace of a reducing substance. It **digests** rapidly in pepsin-HCl; and very slowly in a tryptic mixture.

A NEW METHOD FOR DETECTION OF SMALL AMOUNTS OF CARBON DIOXIDE.

By SHIRO TASHIRO.

(From the Laboratory of Physiology, University of Chicago.)

Making use of the principle which Dr. McCoy and I discovered, I have constructed two new pieces of apparatus which not only detect CO_2 in amounts as small as 0.0000001 gram, but also estimate it with quantitative accuracy. The first apparatus consists of two chambers, the upper one being used for the qualitative detection of the gas, the lower for the quantitative estimation. The second apparatus is used for the combined purposes of qualitative, quantitative and comparative estimation of CO_2 productions from various biological specimens. Unlike any other method for analysis of a gas in small quantity, the new method requires no correction nor precaution against the effects of temperature and pressure variations.

**A STUDY OF THE CHEMICAL CHANGES OCCURRING IN
MEATS DURING THE PROCESS OF DRYING BY THE
VACUUM METHOD.***

BY L. H. DAVIS AND A. D. EMMETT.

(From the Department of Animal Husbandry, University of Illinois.)

The average percentage values for the analysis of five samples of fresh meat and of the corresponding samples of desiccated meat, prepared by Shackell's modification of the Benedict-Manning method were, respectively: 26.46 and 98.21 for dry substance by the vacuum method over sulphuric acid; 26.38 and 96.55 for dry substance by heating in an air oven at 102° C; 2.77 and 10.14 for fat on the vacuum-dried samples; 3.21 and 9.70 for fat on the oven-dried samples; 3.464 and 13.124 for total nitrogen; 0.795 and 2.769 for total water soluble nitrogen; 0.420 and 1.182 for heat coagulable nitrogen; 0.021 and 0.107 for proteose nitrogen; 0.122 and 0.667 for creatine nitrogen; 6.176 and 19.711 for total soluble solid and 0.78 and 3.14 for total soluble ash.

On the dry basis (vacuum method), the average percentage composition of the fresh and desiccated meats was respectively: 10.22 and 10.31 for fat; 13.138 and 13.368 for total nitrogen; 3.011 and 2.835 for total soluble nitrogen; 1.591 and 1.204 for heat coagulable nitrogen; 0.080 and 0.109 for proteose nitrogen; 21.94 and 19.96 for total soluble solids and 2.90 and 3.18 for total soluble ash.

The forms of nitrogen expressed in per cent of the total gave for the fresh and desiccated meats the following respective percentage values: the total soluble, 22.95 and 21.17; the heat coagulable, 12.13 and 8.98; and the proteose, 0.62 and 0.82.

Calculating the data for the fresh and desiccated meats to the dry basis using the two values for dry substance, the vacuum and the oven-heated—the results agree quite closely for the various constituents, the greatest differences being in the fat as was to be expected.

* Read by title.

MUSCLE CREATINE: DIALYSIS OF CREATINE FROM DOG MUSCLE.¹⁰

(PRELIMINARY REPORT.)

BY H. T. LEO AND PAUL E. HOWE.

(From the Laboratory of Physiological Chemistry, University of Illinois.)

A study was made of the dialysis of creatine from muscle under conditions which would tend to throw light upon the form in which this substance is held in the tissues. The experiments of Urano were repeated, using dog-muscle (a) in the form of bundles, (b) ground and placed loosely or packed tightly in collodion bags. Dialysis was commenced immediately after the removal of the muscle from the animal or after it had remained for 24 hours on ice. Each preparation was dialyzed for 2, 2, 2, 12, and 24 hours against 200 cc. portions of Ringer's solution, distilled water, 0.9 per cent NaCl, 1.8 per cent NaCl, 5 per cent NaCl, Ringer's solution + HCl (to make 0.13 per cent), 0.9 per cent NaCl + HCl, 0.13 per cent HCl, and 70 per cent alcohol.

The data indicate that creatine dialyzed most readily from those preparations which were in the loosely-packed, finely-divided form and least, from the more solid preparations, as the muscle bundles and the firmly-packed ground muscle. Dialysis experiments conducted with those substances which should tend to increase the rate of dialysis gave variable results. The action of HCl upon ground muscle was to cause a slower rate of diffusion, but when added to the 0.9 per cent NaCl solution an increased rate of dialysis occurred. The action of the acid upon muscle bundles caused an increase in the rate of diffusion. Alcohol appears to increase the rate of dialysis from muscle bundles but the effect is not equal to that obtained with the HCl nor with the ground muscle in salt solution. Approximately the same results were obtained with the Ringer's solution as with the 0.9 per cent NaCl solution. The hypertonic salt solutions gave a more gradual rate of dialysis, although the ultimate effect was the same. When the results from the various experiments are plotted, the form of the curve is the same (hyperbolic).

The results indicate that simple diffusion experiments do not appear to offer any definite evidence as to the manner in which creatine is held in muscle.

¹⁰Read by title.

STUDIES ON SULPHUR METABOLISM. I. THE URINARY SULPHUR PARTITION IN VARIOUS DISEASES.¹¹

BY N. STADTMÜLLER, M. KAHN AND J. ROSENBLOOM.

(From the German Hospital, New York, and the Laboratory of Biochemistry of the University of Pittsburgh, Pittsburgh, Pa.)

We have studied the urinary sulphur partition (total sulphur, sulphate-sulphur, ethereal sulphate-sulphur, inorganic sulphate-sulphur and neutral sulphur) in the following diseases:

Diabetes mellitus, 10 cases; carcinoma, 13 cases; nephritis, 2 cases; pneumonia, 2 cases; lead poisoning, 2 cases; bronchial asthma, 1 case; chronic appendicitis, 2 cases; hepatic abscess, 1 case; hepatitis, 2 cases; cholelithiasis, with biliary fistula, 1 case; typhoid fever, 1 case; chronic myocarditis, 1 case; hypopituitarism, 1 case; gastroptosis and gastric dilatation, 1 case.

The urine has been analyzed daily for periods varying from three days to two weeks, and we have found high proportions of neutral sulphur in all the cases of diabetes (except one). The neutral sulphur was also high in all the cases of carcinoma, in one case of nephritis and pneumonia and in the one case of hypopituitarism.

THE METABOLIC END-PRODUCTS OF THE LIPOID NITROGEN OF EGG YOLK.¹¹

By E. V. MCCOLLUM AND H. STEENBOCK.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin.)

A pig of 49 pounds was fed during seven days on a ration of 220 grams of dry egg yolk and 85 grams of starch per day. The nitrogen intake was 11.65 grams per day. Of this 0.65 gram was lipid nitrogen. The total N of the urines varied from 5.029-6.098 grams. From 30 to 40 per cent of the total nitrogen could be removed by the Folin method for ammonia. Urine determinations by the Benedict and by the Henriques and Galloway methods gave fairly close agreement, and indicated that only 35-45 per cent of the total nitrogen was present as urea.

¹¹ Read by title.

Of the nitrogen removed by aspiration, 5.2–16.7 per cent could not be absorbed by mercury oxide according to Erdmann's method. By this method the urines were found to contain on an average 0.2989 gram of nitrogen as substituted amines. It is evident that demethylation of substituted amines is not readily accomplished in the body of the pig.

THE OCCURRENCE OF URIC ACID IN BLOOD.¹²

By OTTO FOLIN AND W. DENIS.

A NEW METHOD FOR DRYING TISSUES AND FLUIDS.¹²

By JACOB ROSENBLOOM.

METABOLISM OF A DWARF.¹³

By GRAHAM LUSK.

THE FATE OF FATTY ACIDS IN DIABETIC ORGANISMS.¹²

By A. I. RINGER.

ON THE SECRETION OF PURE ACID BY THE KIDNEY.

By A. B. MACALLUM AND W. R. CAMPBELL.

HYPERTROPHY AND HYPERPLASIA OF THE PARATHYROID IN BIRDS.

By D. MARINE.

QUANTITATIVE OXIDASE MEASUREMENTS.¹⁴

By H. H. BUNZEL.

THE REGULATING FUNCTION OF AMYLASE BY THE FUNGUS GLOMERELLA.¹⁴

By HOWARD S. REED.

SYMPOSIUM.

SOME RECENT APPLICATIONS OF PHYSICAL CHEMISTRY IN BIOLOGY.

A. B. MACALLUM.—Surface tension.

L. J. HENDERSON.—The control of neutrality in the animal body.

A. S. LOVENHART.—The physical chemistry of enzyme action.

¹² Published in full in *Journal of Biological Chemistry*, xiv, February, 1913.

¹³ Published in full in *Journal of Biological Chemistry*, xiii, January, 1913.

¹⁴ Read by title.



TANNIC ACID FERMENTATION. I.

By LEWIS KNUDSON.

(From the Laboratory of Plant Physiology, Cornell University, Ithaca, N. Y.)

(Received for publication, January 30, 1913.)

I. PREFACE.

CORRECTIONS.

On page 135, Vol. XIV, No. 2, March 1913, fourth line from the bottom for *glucose* read *glycogen*.

On page 136 of the same number, fifth line from the bottom, omit *to*.

subsequent paper.

These investigations were begun at the suggestion of Prof. B. M. Duggar and prosecuted in his laboratory. It is a pleasure here to express my thanks for the advice, kindly criticism and assistance, which he has so generously given.

II. INTRODUCTION.

Chemical nature of tannic acid. Wagner¹ has grouped the tannins into a "physiological" and a "pathological" series, the latter including, as most important, the tannin of oak galls as

¹ R. Wagner: Beiträge zur Kenntnis und zur quantitativen Bestimmung der Gerbsäuren, *Zeitschr. f. anal. Chem.*, v, pp. 1-10, 1866.



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I. PREFACE.

During 1907-1908, a preliminary investigation of the conditions of tannic fermentation was made by the writer with the purpose of improving the practical methods involved. In the course of this preliminary investigation a number of interesting observations were made, justifying a further study of the conditions of the fermentation and of the relation of various organisms to the process. With the progress of the investigation other phases suggested themselves, until ultimately four distinct but correlated parts of the subject were experimentally studied. Part I, here reported, includes chiefly: (1) the toxicity of tannic acid for various fungi; (2) a comparison of the organism *Aspergillus niger* and *Penicillium* sp. in the fermentation of tannic acid; (3) the conditions and influence of various factors on the fermentation process. Part II is concerned primarily with the influence of nutrition on the production of the enzyme tannase, and will be reported in a subsequent paper.

These investigations were begun at the suggestion of Prof. B. M. Duggar and prosecuted in his laboratory. It is a pleasure here to express my thanks for the advice, kindly criticism and assistance, which he has so generously given.

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well as the tannin of sumach and chestnut. The pathological tannins are hydrolyzed by boiling with acids or through the action of the enzyme tannase, gallic acid resulting. For other distinguishing characteristics of the tannins Trimble's² and Proctor's³ treatises may be consulted, and for a discussion of the diverse views held regarding their chemistry reference should be made to special papers on the subject.

This paper is concerned with the fermentation of the tannin from oak galls, which is frequently termed gallotannic acid.

History of tannic acid fermentation. Scheele⁴ found in 1786 that gallic acid was present in the gall nuts. Robiquet⁵ attributed the fermentation of the gall nuts to a ferment within the gall nut. Laroque⁶ considered the formation of gallic acid from tannic acid to be due either to a ferment or to oxidation. He further found that various toxic substances could inhibit the fermentation. Ed. Robiquet⁷ showed that the tannic acid was transformed during the fermentation and he believed the transformation to be due to the ferment pectase which he extracted from the gall nuts. Wittstein⁸ stated that beer yeast aided tannin fermentation by fermenting the sugars and other products present. Van Tieghem⁹ was the first, however, to demonstrate that the formation of gallic acid during fermentation is due to the action of fungus organisms, and not to enzymes pre-existing in the galls, nor to oxidation by the air. He stated further that the organisms were *Penicillium glaucum* and a new organism which he named *Aspergillus niger*. He found that if the growth was submersed, the tannic acid was converted into gallic acid and glucose, the glucose being gradually used up, the gallic acid remaining. He stated further that if the growth was on the surface, sporulation and greater growth occurred and that the tannic acid was destroyed directly, the slight hydrolysis being due to submerged mycelium, the resulting glucose and gallic acid being then assimilated.

² H. Trimble: *The Tannins*, Part I, 168 pp.; Part II, 172 pp., 1892.

³ H. K. Proctor: *Leather Industry. Laboratory Book*, 2nd edition, 450 pp., 1908.

⁴ Quoted from H. Trimble: *loc. cit.*

⁵ M. Robiquet: Faits pour servir à de l'acide gallique, *Ann. de chim. et de phys.*, 2^e serie, lxiv, pp. 385-409, 1837.

⁶ A. Laroque: Neue Untersuchungen über Gallussäure, *Ann. d. Chem. u. Pharm.*, xxxix, pp. 37-100, 1841.

⁷ Ed. Robiquet: Recherches sur la fermentation gallique, *Ann. de chim. et de phys.*, 3^e serie, xxxix, pp. 453-460, 1853.

⁸ Wittstein: *Jahresber. über die Fortschritte der Chemie*, 1853, p. 435.

⁹ Ph. VanTieghem: Sur la fermentation gallique, *Compt. rend. de l'Acad. des Sci.*, lxxv, pp. 1091-95, 1867.

Müntz¹⁰ found that fermentation occurred through the action of *Penicillium glaucum*. Fernbach¹¹ grew *Aspergillus niger* in Raulin's solution with the sugar replaced by tannic acid, and then extracted from the organism the enzyme tannase. Pottevin,¹² in a similar manner and at the same time, extracted the enzyme tannase from the same fungus. He noted also that the enzyme was developed when *Aspergillus niger* was grown on Raulin's solution with the sugar replaced by gallic acid. He stated that the tannase acted on tannate of gelatin and also hydrolyzed methyl salicylate and ethyl salicylate.

Manea¹³ showed that synthetically prepared digallic acid was not split up by *Aspergillus niger* and *Penicillium glaucum* into gallic acid, and therefore concluded that the hydrolyzable tannin of the gall nut could not be a digallic acid. The latter in high concentrations was toxic to the organism. Further, Manea estimated quantitatively the digallic and tannic acid used by each organism. In a study of the fermentation process he employed pure cultures, adding a previously sterilized Raulin's solution rendered strongly acid. The quicker the fermentation, the richer was the yield of gallic acid obtained.

Kunz-Krause¹⁴ found an octyl gallotannoid, $C_{44}H_{40}O_{31}$, which through the action of a mould was transformed to gallic acid.

III. METHODS.

Culture solution. Throughout all the work the culture solutions used were a slight modification of Richards¹⁵ solution or of Czapek's solution.¹⁶ These solutions are designated respectively A and B and are as follows:

¹⁰ Müntz: *Ber. d. deutsch. chem. Gesellsch.*, 1877, p. 1773.

¹¹ A. Fernbach: Sur la tannase, *Compt. rend. de l'Acad. des Sci.*, cxxxi, pp. 1214-15, 1901.

¹² H. Pottevin: La tannase. Diatase dedoublant l'acide gallotannique, *Compt. rend. de l'Acad. des Sci.*, cxxxi, pp. 1215-17, 1901.

¹³ A. Manea: *Sur les acides gallotanniques et digallickes*. These, Geneva, 1904. (Cited from Lafar: *Handb. d. technische Mykologie*, I, p. 663.)

¹⁴ Kunz-Krause: Fragmente zu einer Monographie d. Tannoide, *Pharm. Centralbl.*, Halle, 1898. (Cited from Lafar: *Handb. d. technische Mykologie*, I, p. 662.)

¹⁵ H. M. Richards: Die Beeinflussung des Wachstums einiger Pilze durch chemische Reize, *Jahrb. f. wiss. Bot.*, xxx, pp. 665-688, 1897.

¹⁶ Quoted from A. W. Dox: Intracellular Enzymes of *Penicillium* and *Aspergillus*, U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120, 70 pp., 1910.

SOLUTION A.		SOLUTION B.	
KNO ₃	1.0 gram.	MgSO ₄	0.5 gram.
KH ₂ PO ₄	0.5 gram.	K ₂ HPO ₄	0.1 gram.
MgSO ₄	0.25 gram.	KCl.....	0.5 gram.
Distilled H ₂ O.....	100 cc.	NaNO ₃	2.0 gram.
		Distilled H ₂ O.....	1000 cc.

The source of carbon was cane sugar, tannic acid or gallic acid, either alone or supplementing each other, depending upon the experiment. A 10 per cent concentration of sugar was employed, as experience has shown that the better growth is secured with this concentration than with the lower concentration. This fact is developed in a subsequent table.

Methods of inoculation. In all of the fermentation experiments the method of inoculation employed was that proposed by Hasselbring.¹⁷

Methods of analysis. The volumetric method of Dreaper¹⁸ was used for some experiments, but for most of the work the volumetric method proposed by Jean¹⁹ was used. Both methods have imperfections, but they are approximately accurate. In all cases analyses were checked by duplicate determinations and usually by more.

Method of washing and weighing the fungus felt. For the experiments, the results of which are included in tables I and II, the method used in washing the felt free from gallic and tannic acid was as follows:

The felt was removed by means of a bent needle and floated on distilled water, the water being renewed until it gave no further coloration. In order to secure the submersed growth, the solution was poured into a cylinder and the submersed growth, which now usually floated on the surface, was then removed by needles. All of the mycelium was then placed in a crucible, which had been brought to constant weight at 105°, heated for five hours at the same temperature and then weighed. This method, as well as the use of filter paper, possesses obvious disadvantages as well as being inaccurate. For most of the work, therefore, the following method was used: The Gooch

¹⁷ H. H. Hasselbring: Carbon Assimilation of *Penicillium*, *Bot. Gazette* xlv, pp. 176-193, 1908.

¹⁸ W. P. Dreaper: Estimation of Tannic and Gallic Acid, *Chem. News*, xc, pp. 111-112, 1904.

¹⁹ F. Jean: Die Bestimmung des Tannins und der Gallussäure, *Chem. Centralbl.*, 1900, pp. 1107-08.

filter was prepared in the usual manner, as employed in quantitative chemical analysis, and filtration was made by means of the Gooch funnel with suction. The original solution was first decanted into the Gooch crucible. The felt was then washed in the flask four or five times with distilled water at room temperature; or, if gallic acid had been precipitated, warm water was used. The washing of the felt continued until the wash water was perfectly clear. The felt was then placed in the Gooch crucible, the flask again washed and the wash water poured into the Gooch crucible. The advantages of the method consist in the rapidity of the filtration and the accuracy which results from the thorough washing, which latter is important when the culture solution is to be analyzed and absolute weight of mycelium is to be obtained. It is an especially accurate method of securing all the fungous mycelium, and by exercising a little care there is no noticeable loss of spores.

IV. TOXICITY OF TANNIC ACID FOR CERTAIN FUNGI.

In the literature of tannic acid fermentation only two organisms are mentioned as possessing the property of effecting this fermentation; these are *Aspergillus niger* and *Penicillium glaucum*. In order to determine whether other organisms are capable of effecting the transformation, a considerable number of filamentous fungi were carefully tested with respect to their ability to grow in tannic acid solutions.

As a nutrient medium a bean decoction was made by boiling 1 liter of laboratory preserved beans with a liter of tap water. The juice was then filtered off and diluted to 2 liters. With this decoction as a solvent, four concentrations of tannic acid were made; namely, 0.25 per cent, 2 per cent, 5 per cent and 10 per cent. Test tubes were employed as culture vessels, to each of which were added 10 cc. of the solution. Small wads of filter paper were added to afford a solid substratum. The tubes were prepared in duplicate, sterilized, inoculated and kept at room temperature. They were examined at intervals and the final observations made at the end of two weeks are recorded in table A.

It is especially noteworthy that the 5 per cent permitted the growth of only one-third of the organisms, while in the 10 per cent solution only *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* sp. are able to grow. A separate experiment indicated that *Aspergillus oryzae* could withstand 10 per cent tannic acid.

An experiment was also made to determine if any of these organisms could utilize tannic acid as a source of carbon. Solution B with 11.6 grams of tannic acid per 100 cc. of solution was used.

TABLE A.

ORGANISM	CHARACTER OF GROWTH			
	0.25 per cent	2 per cent	5 per cent	10 per cent
1. <i>Penicillium brevicaulis</i>	good	good	good	
2. <i>Penicillium camemberti</i>	good	good	very slight	
3. <i>Penicillium claviforme</i>	good	good	good	
4. <i>Penicillium duclauxii</i>	good	good	none	
5. <i>Penicillium granulatum</i>	good	good	good	
6. <i>Penicillium italicum</i>	good	slight	slight	
7. <i>Penicillium lilacinum</i>	good	slight	none	
8. <i>Penicillium purpurogenum</i>	good	none	none	
9. <i>Penicillium sp.</i>	good	good	good	good
10. <i>Aspergillus flavus</i>	good	good	good	good
11. <i>Aspergillus niger</i>	good	good	good	good
12. <i>Trichoderma lignorum</i>	good	none	none	
13. <i>Mucor circinelloides</i>	good	slight	none	
14. <i>Mucor rouxii</i>	good	slight	none	
15. <i>Mucor spinosus</i>	good	none	none	
16. <i>Polyporus sulphureus</i>	good	none	none	
17. <i>Polyporus resinousus</i>	good	none	none	
18. <i>Fomes megaloma</i>	no growth	none	none	
19. <i>Chaetomium sp.</i>	good growth	none	none	
20. <i>Chaetostylum sp.</i>	good growth	none	none	
21. <i>Stysanus sp.</i>	good growth	none	none	
22. <i>Cephalothecium roseum</i>	good	none	none	
23. <i>Circinella umbellata</i>		good	none	

Erlenmeyer flasks of 150 cc. capacity were employed, and in each were placed 50 cc. of the culture solution. After sterilization these flasks were inoculated and maintained at room temperature. In addition to the above list of organisms the following were tested: *Aspergillus oryzae*, *Nectria ipomoeae*, *Fusarium oxysporum*, *Phycomyces nitens* and *Stilbella sp.* Of all organisms tested only *Aspergillus niger* and *Penicillium sp.*²⁰ developed. Duplicate cultures of all these organisms on Chinese galls gave similar results, except in this case *Aspergillus flavus* produced a very slight growth.

VanTieghem²¹ found that both *Aspergillus niger* and *Penicillium glaucum* could withstand a saturated solution of tannic acid, and

²⁰ One other species of *Penicillium*, as indicated in the appendix, is able to develop upon a 10 per cent tannic acid solution.

²¹ *Loc. cit.*

the fact that both develop in moistened gall nuts, which contain per dry weight 60 per cent of tannic acid, is evidence that for these two organisms the tannic acid is not toxic.

Toxicity of tannic acid for Aspergillus flavus and Aspergillus oryzae. An experiment was conducted to determine the growth at various concentrations of tannic acid with and without 10 per cent cane sugar. In one case tannic acid (Merck's tested reagent) was added to solution B; in the other, tannic acid + 10 per cent sugar was used. Test-tube cultures with 15 cc. of the solutions were employed. The results in general showed that these two fungi develop normally in the presence of 2.5 per cent tannic acid, but greater concentrations decrease the rate of germination and inhibit the growth. In the 15 per cent concentrations of tannic acid, after nine days, only one-third of the surface was felted. Up to 7.5 per cent concentration the entire surface was felted.

Conclusion and discussion. The experiments on the toxicity of tannic acid indicate that of all the organisms tested, *Aspergillus niger* and *Penicillium sp.*²² are best adapted for the tannic acid fermentation. These two organisms were, therefore, selected for more detailed investigation, though the other two organisms previously mentioned were also reserved for further study.

Since the above experiments were made, a bulletin has appeared on the toxicity of tannin by Cook and Taubenhaus.²³ The majority of a large number of parasitic organisms tested by them with respect to the toxicity of tannin show retardation of growth at from 0.1 per cent to 0.8 per cent of tannin. The few saprophytic forms tested exhibit a more marked resistance. My own experiments indicate also that the saprophytic forms can withstand relatively higher concentrations of tannic acid than the parasitic forms.

²² See appendix for description of this organism.

²³ M. T. Cook and J. J. Taubenhaus: The Relation of Parasitic Fungi to the Contents of the Cells of the Host Plant, Delaware Agric. Exp. Station, Bulletin 91, 77 pp., 1911.

V. COMPARISON OF ORGANISMS AND INFLUENCE OF TIME AND GROWTH ON FERMENTATION.

Comparison of organisms. Employing an infusion of gall nuts containing 10.5 per cent tannic acid, VanTieghem²⁴ found that the fermentation was completed by *Aspergillus niger* in six days and by *Penicillium glaucum* in eight days, when the temperature of incubation was 35° and the supply of oxygen was limited by keeping the flask stoppered. In the weaker concentrations, *Penicillium glaucum* fermented the tannic acid more vigorously. Since in VanTieghem's experiments the temperature was rather high and the conditions of growth approached anaerobic conditions, it was believed that a better comparison could be made by supplying the optimum conditions for the growth of each. In all the natural fermentations (in unsterilized solution) of the tannic acid, *Aspergillus niger* always developed first and then *Penicillium sp.* So marked is this succession that by exposing a nutrient solution of 10 per cent tannic acid to the air a pure culture of *Aspergillus niger* may usually be obtained. This suggests that *Aspergillus niger* may possess the greater fermentative capacity, but an experiment was required.

In the first experiment solution A and tannic acid were used, 50 cc. of the solution being placed in Erlenmeyer flasks of 150 cc. capacity. The flasks were sterilized for forty-five minutes at 115°, inoculated, and kept for two weeks at a temperature of from 18°-28°, the average being close to 24°. The felts were then removed, thoroughly washed, and the wash water added to the culture solution, the solution being then brought up to 500 cc. volume and analyzed according to Dreaper's²⁵ method. The figures below are for *Aspergillus niger*, the averages of six cultures, while for *Penicillium sp.* the averages of five cultures are given.

TABLE I.

ORGANISM	TANNIC ACID IN CULTURE SOLUTION	LOSS IN TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS OF GALLIC ACID	NET WEIGHT OF FUNGUS
	grams	grams	grams	grams	gram
<i>Aspergillus niger</i>	0.228	1.244	0.200	2.752	0.848
<i>Penicillium sp.</i>	0.332	1.140	1.780	1.172	0.537
Check.....	1.472		2.952		

²⁴ *Loc. cit.*²⁵ *Loc. cit.*

The loss in gallic acid indicates that this substance is used by the organisms as a source of carbon, which fact agrees with the observations of VanTieghem²⁶ and Pottévin.²⁷ According to VanTieghem, when growth occurs on the surface the tannic acid is utilized directly without previous conversion into gallic acid. There is no evidence for this assumption. If the tannic acid is not utilized directly, and it probably is not, then *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium sp.* for in the *Penicillium* culture more tannic acid remained and the decrease in gallic acid was only 39 per cent. The larger gallic acid content of the *Penicillium* culture is related to the smaller amount of growth and not to the greater practical efficiency as a fermentative organism, and this point is more apparent from later work.

In table II there are given, separately, data for four of the *Penicillium* cultures. This table emphasizes a general relation between the amount of growth and the extent of fermentation; furthermore, the disappearance of gallic acid is correlated with increased growth.

TABLE II.

CULTURE NO.	TANNIC ACID IN CULTURE SOLUTION	LOSS IN TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS IN GALLIC ACID	WEIGHT OF FUNGUS
	grams	grams	grams	grams	gram
Check	1.472		2.952		
1	1.216	0.156	2.396	0.556	0.295
2	0.140	1.332	2.240	0.700	0.450
3	0.152	1.320	1.760	1.192	0.550
4	0.100	1.372	1.308	1.644	0.700

Culture No. 1 seems to indicate that the tannic acid transformation is dependent upon the amount of growth, for only a small amount of tannic acid was transformed, but a greater growth during the same period in the other cultures resulted also in almost complete transformation.

Since the most economical production of gallic acid is dependent upon the amount of growth, and growth amount is a function of time, temperature, aeration, and nutrition, then these factors should be important. Growth is emphasized because with it

²⁶ *Loc. cit.*²⁷ *Loc. cit.*

the amount of the enzyme is probably correlated, at least with respect to *Penicillium* sp.

Culture No. 1 shows a small decrease in tannic acid and a high decrease in gallic acid. In culture No. 2 the loss of tannic acid is greater than loss of gallic acid. The probable explanation is that *Penicillium* sp. utilizes first the gallic acid and then transformation of the tannic acid occurs. In culture No. 1 the smaller growth has been at the expense of tannic acid. It does not seem possible to demonstrate that tannic acid is not directly utilized but it seems probable that it must be converted into gallic acid.

Influence of duration and extent of growth and comparison of organisms. In order to determine the yield of gallic acid at different intervals, and so to note the influence of growth and duration on the fermentation, an experiment was made as follows: Solution B was used and to it were added 7.5 grams of Merck's purified tannic acid per 100 cc. of solution. The concentration of the tannic acid was such that it came within the limits, as found by Van Tieghem,²⁸ most favorable for *Penicillium glaucum*. For the investigation 50 cc. of the culture solution were placed in Erlenmeyer flasks of 150 cc. capacity. The cultures were sterilized for fifteen minutes at 5 pounds' pressure and then inoculated according to the method described. The cultures were kept in an incubator at 31° and, at intervals, as indicated, duplicate cultures were taken for analyses. The mycelium was removed by filtering with suction through the Gooch crucible, and it was then washed with warm water to dissolve all adhering gallic acid. The solution was then brought up to 500 cc. and analyzed according to the method of Jean.²⁹ The results of the experiment are given in Table III.

In culture No. 8 the amount of gallic acid decrease was 0.126 gram and the tannic acid decrease only 0.069 gram, recalling the case of No. 1, table II. *Penicillium* sp., it seems, therefore utilizes the gallic acid first, and then the secretion of enzyme involves the transformation of the tannic acid. In all of the succeeding cultures the tannic acid decrease is greater than the increase of gallic acid, and this is to be accounted for, again, not by a direct utilization of tannic acid but by the fact that the tannic acid is first converted into gallic acid; and the constant increase of the

²⁸ Loc. cit.

²⁹ Loc. cit.

TABLE III.
Organism, Aspergillus niger.

CULTURE NO.	DURATION	TANNIC ACID IN CULTURE SOLUTION	LOSS OF TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS OR GAIN OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	days	grams	grams	grams	grams	gram
1	4	0.750	0.853	2.584	+0.484	0.0314
2	6	1.057	0.546	2.191	+0.091	0.1568
3	8	0.252	1.351	1.814	-0.286	0.1946
4	10	0.306	1.297	1.001	-0.499	0.4688
5	12	0.160	1.443	1.460	-0.640	0.4933
6	16	0.159	1.444	1.406	-0.694	0.5137
7	28	0	1.603	0.898	-1.202	0.6345
Check		1.603		2.100		

Organism, Penicillium sp.

8	4	1.534	0.069	1.974	-0.126	0.0157
9	6	1.330	0.273	2.191	+0.091	0.0800
10	8	1.296	0.305	1.769	-0.331	0.1458
11	10	0.750	0.853	1.669	-0.431	0.3031
12	12	0.427	1.176	1.333	-0.767	0.3880
13	16	0.597	1.006	1.530	-0.670	0.4096
14	28	0.106	1.497	1.277	-0.823	0.4284

gallic acid prevents the utilization of the gallic acid from being made manifest.

If the *Aspergillus niger* and *Penicillium sp.* cultures are compared, one finds that the tannic acid in the *Aspergillus* culture is transformed to the extent of 81 per cent by the tenth day; whereas in the *Penicillium* cultures this transformation, for the corresponding time, is only 53.3 per cent of the tannic acid. Moreover, by the fourth day the gallic acid had increased in culture No. 2 by nearly 0.5 gram, while in culture No. 8, for a corresponding time, there was a decrease of gallic acid. With this concentration and temperature, therefore, the *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium sp.*

If now the time factor and amount of growth be examined in their relation to the gallic acid, it is found that with the above solutions and under the specified conditions, the gallic acid decreases after the sixth day and is utilized in the further metabolism of the organism.

Comparison of fermentative capacity in an infusion of gall nuts. For the investigation an extraction of the gall nuts was made as follows: 1800 grams of the Aleppo gall nuts were placed in one jar and the same quantity of Chinese nuts in another. To each were added 3 liters of tap water, and the extraction was allowed to continue for five days, when the extract was filtered. To each jar were added again 2 liters of water, and after one day the extracts were filtered and combined with the previous filtrates. Then the two extracts from the Chinese and Aleppo galls were mixed. After a second filtration the solution was ready for the culture vessels. For this purpose cultures were made in the usual way, using Erlenmeyer flasks of 150 cc., each, with 50 cc. of the infusion.

TABLE IV.

CULTURE NO.	DURATION days	TANNIC ACID IN CULTURE SOLUTION grams	LOSS OF TANNIC ACID grams	GALLIC ACID IN CULTURE SOLUTION grams	GAIN OF GALLIC ACID grams	DRY WEIGHT OF FUNGUS gram
Check		1.637		1.797		
<i>Aspergillus niger.</i>						
1	4	0.545	1.092	2.752	0.955	
2	6	0.218	1.419	3.218	1.421	0.197
3	8	0	1.637	2.500	0.703	0.262
4	12	0	1.637	2.527	0.730	0.171
5	20	0.206	1.431	2.527	0.730	0.205
6	56	0	1.637	2.527	0.730	0.148
<i>Penicillium sp.</i>						
7	4	1.330	0.307	2.050	0.253	0.043
8	6	0.545	1.092	2.627	0.730	0.157
9	8	0.409	1.128	2.387	0.590	0.110
10	12	0.034	1.603	2.471	0.674	0.171
11	20	0.085	1.552	2.457	0.660	0.117
12	56	0.152	1.415	2.247	0.450	0.151

A comparison of the two organisms, as regards their fermentative capacity, shows again that *Aspergillus niger* is a more efficient organism. Note especially that in four days the *Aspergillus* cultures exhibit an increase of gallic acid nearly four times as great as that in the *Penicillium* cultures, and all of the tannic acid had been converted in the former by the eighth day, while in the *Peni-*

cillium cultures only 70 per cent was converted in the same time. Not only does *Aspergillus niger* produce a more rapid fermentation, but also a greater production of gallic acid is effected; for, as indicated, the maximum yield of gallic acid in the *Aspergillus* cultures is twice that in the *Penicillium* cultures, and this in spite of the fact that a greater weight of *Aspergillus niger* is produced. Furthermore, in the *Penicillium* cultures there occurs a decrease of the gallic acid even when considerable tannic acid is still present in the culture solution.

In order to understand these differences between *Aspergillus niger* and *Penicillium sp.*, the composition of the infusion must first be considered and an idea of this may be obtained from constituents of the gall nuts. In addition to tannic acid, the gall nuts contain (Guibert²⁰) gallic acid, chlorophyll, starch, gums, sugar, proteins and various inorganic salts and other compounds. A water extract of the gall nuts would contain in solution and suspension a certain amount of most of these substances, and the subsequent sterilization would probably result in transformations which would make certain of the organic compounds more available.

Aspergillus niger is an omnivorous organism in its relation to the utilization of carbon compounds. The growth of this organism for the first few days is probably at the expense of the other organic substances present, and the gallic acid, in this case, accumulates. All the facts indicate that while *Aspergillus niger* is utilizing the organic compounds other than tannic acid, it secretes the enzyme tannase (this point will be further developed in a separate paper), and consequently the transformation of the tannic acid goes on, and gallic acid accumulates. When the other organic compounds are exhausted, the gallic acid is utilized, and then the decrease begins. As shown previously, *Penicillium sp.* tends to utilize the gallic acid before it transforms the tannic acid, for gallic acid is a favorable nutrient carbon compound for this organism. Furthermore, as I will show in a later paper, the presence of the other organic compounds may decrease the secretion of the enzyme tannase by *Penicillium sp.*; and since the utilization of the gallic acid exceeds the formation of this substance, there results a decrease

²⁰ Quoted from H. Trimble: *loc. cit.*

of the gallic acid despite the fact that tannic acid is present in solution.

A comparison of tables III and IV is of interest. The experiment with a synthetic solution and the other with the infusion were conducted at the same time and under identical conditions as regards sterilization, inoculation and incubation. Moreover, the tannic acid and the gallic acid content of the culture solutions are nearly the same.

In table V, only the duration of experiment, decrease of tannic acid, the loss or gain of gallic acid and the dry weights of the fungus produced, are included.

TABLE V.

DURATION days	SOLUTION B + 1.608 GRAMS TANNIC ACID + 2.100 GRAMS GALLIC ACID			INFUSION OF GALL NUT: TANNIC ACID = 1.627 GRAMS GALLIC ACID = 1.797 GRAMS		
	LOSS OF TANNIC ACID	LOSS OR GAIN OF GALLIC ACID	DRY WEIGHT OF FUNGUS	LOSS OF TANNIC ACID	GAIN OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	grams	gram	gram	grams	grams	gram
<i>Aspergillus niger.</i>						
4	0.753	+0.484	0.031	1.092	+0.955	
6	0.546	+0.091	0.0156	1.419	+1.421	0.197
8	1.351	-0.286	0.194	1.637	+0.703	0.262
12	1.443	-0.640	0.493	1.637	+0.730	0.171
16	1.444	-0.694	0.513			
20				1.637	+0.730	0.148
<i>Penicillium sp.</i>						
4	0.069	-0.126	0.015	0.307	+0.253	0.043
6	0.273	+0.091	0.080	1.092	+0.760	0.157
8	0.305	-0.331	0.145	1.128	+0.590	0.110
12	1.176	-0.767	0.188	1.603	+0.674	0.171
16	1.016	-0.670	0.4096			
20				1.415	+0.450	0.151

It is interesting to note that in the synthetic solution the gallic acid decreased after the eighth day, while in the gall nut infusion it showed at that time a marked increase, and this increase was maintained thereafter. In the synthetic solution on the eighth day there was a loss of 0.286 gram of gallic acid, while in the gall nut infusion there was a gain of 0.703 gram of gallic acid, and in

the latter the tannic acid was completely transformed. The difference in the amount of tannic acid transformed was not sufficient, however, to account for the loss of gallic acid in the synthetic solution and the marked increase in the infusion culture. Despite the greater weight of the fungus this increase of gallic acid was more in the infusion culture. A similar condition existed at the end of six days. The explanation of this point seems to be found in the fact that the infusion cultures contain various organic compounds which are utilized in place of the gallic acid; that is, elected in preference. At the end of four days probably none of the gallic acid has been assimilated. The subsequent decrease in gallic acid was due to its use after the exhaustion of more favorable organic nutrients. After the sixth day in the infusion culture there was no further growth of the organisms and no decrease in the gallic acid. The growth was less than one-half of that in the synthetic cultures, probably due to the lack of inorganic nutrients, although the presence of injurious metabolic products might also have been a factor, as the organic food supply was by no means exhausted. The conditions which obtained for the *Aspergillus niger* cultures apply also to those of *Penicillium* sp.

Another point of interest brought out by the comparison is, the more rapid fermentation in the infusion than in the synthetic solution. In the *Aspergillus* cultures the tannic acid was completely transformed in the gall nut infusion by the eighth day, when the weight of the fungus was 0.262 gram. In the synthetic cultures transformation was not complete by the sixteenth day, when the weight of dry mycelium was 0.513 gram. In the *Penicillium* cultures, also, the results are comparable.

In another experiment in which a gall nut infusion was used containing 2.04 grams of tannic acid and 0.56 gram of gallic acid per 50 cc., at room temperature, there was maintained for thirty days an increase of gallic acid. Practically all of the fermentation occurred before the eleventh day, and the gallic acid was protected by the other organic substances.

Since the experiments with the infusion cultures indicated that the gallic acid was protected to a certain extent by the election of other organic substances, it was determined to try the addition of sugar to the culture solution with respect to its effect on the election of foods and hence on the yield of gallic acid.

VI. INFLUENCE OF THE ADDITION OF SUGAR.

Effect of 5 per cent sugar. For these cultures solution A was used to which was added in the one series tannic acid alone, and to the other, tannic acid and cane sugar. Sugar was added at the rate of 5 grams per 100 cc. of solution, and the amount of tannic acid is indicated by the check. The cultures were made in liter flasks, and 500 cc. of the solution were used for each. The flask and contents were sterilized, inoculated and kept in the incubator at a temperature which varied from 27°–30°C. The results of the experiment given in table VI are in all cases obtained from duplicate cultures.

TABLE VI.

CULTURE NO.	DURATION	TANNIC ACID IN CULTURE SOLUTION	LOSS IN TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	GAIN OR LOSS OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	days	grams	grams	grams	grams	grams
SERIES I. <i>Solution A + tannic acid.</i>						
1	10	4.568	8.193	11.095	+6.867	1.42
2	20	0.203	12.558	2.190	–2.038	3.66
3	30	0.050	12.711	0.142	–4.086	4.00
Check		12.761		4.228		
SERIES II. <i>Solution A + tannic acid + cane sugar.</i>						
4	10	0.913	11.848	8.761	+4.533	6.008
5	20	0.812	11.949	3.904	–0.324	10.35
6	30	0.101	12.660	0.143	–4.085	13.10
Check		12.761		4.228		

The results of the experiment seemed at first surprising. Instead of getting a protective action of the sugar with respect to the gallic acid, and thereby an increased yield of gallic acid, the opposite condition seemed to result. The yield of gallic acid was actually less at the end of ten days in the solution containing sugar than in the solution which lacked sugar, even though more of the tannic acid was transformed in the former. At the end of twenty days more gallic acid was left in the cultures of series II than in the corresponding culture solutions of series I, and so a certain protective action of the sugar is evident. At the end of thirty days practically all of the tannic acid and gallic acid had disappeared from

the culture solutions. In explanation of the seeming failure of the sugar to protect the gallic acid, the dry weights of felts produced in the corresponding cultures must be compared. The weight of fungus produced in each culture of series II was, at the end of each period, at least three times as great as the weight of the corresponding cultures of series I. This increased growth and the accompanying increased respiration were sufficient to utilize practically all of the organic-nutrients supplied, usually all of the sugar and some of the gallic acid.

Effect of 10 per cent sugar. Since negative results were obtained as regards the protection of the gallic acid by 5 per cent sugar a new series of cultures was made with 10 per cent sugar in solution B to which was added the tannic acid required. On analysis the solution showed after sterilization 4.171 grams of tannic acid and 2.198 grams of gallic acid per 50 cc. The cultures were incubated at a temperature of 28°, though it dropped occasionally, owing to an imperfect thermostat, to 25° and rose likewise to 32°. The cultures were taken down at definite intervals, the weight of the felts determined, and the analyses of the culture solutions made according to the methods previously described. The results follow in table VII.

The protective action of the sugar is at once evident. Since the concentration of tannic acid here is double that in the experiments which are included in table III, and therefore a comparison of the yields of gallic acid is not possible, yet the great increase of gallic acid and the maintenance of this increase prove that the sugar has been utilized in place of the gallic acid. Here it is obviously not true that the greater the weight the less the gallic acid. Cultures Nos. 4, 5, 6 and 7 all vary in weight, yet the amount of gallic acid in each is approximately the same, and any difference may be due to the imperfect method of analysis. Even at the expiration of thirty-five days no decrease of the gallic acid was evident. It may be concluded, therefore, and further experiments prove, that the 10 per cent sugar protects the gallic acid.

While an increase of gallic acid is maintained in the *Penicillium* cultures, the increase is relatively small and is due to a slower transformation of the tannic acid; practically no transformation of tannic acid occurred until after the fourteenth day. Previous to this time the growth was entirely submersed, but afterwards fructi-

TABLE VII.

CULTURE NO.	DURATION <i>days</i>	GALLIC ACID IN CULTURE SOLU- TIONS <i>grams</i>	INCREASE OF GAL- LIC ACID <i>grams</i>	DRY WEIGHT OF FUNGUS <i>grams</i>
Check		2.198		
<i>Aspergillus niger.</i>				
1	3	4.929	2.731	0.1579
2	6	5.028	2.830	0.4093
3	8	5.533	3.335	0.4135
4	14	*6.320	4.122	0.5148
5	20	*6.320	4.122	0.4470
6	25	*6.292	4.094	0.4820
7	35	*6.348	4.150	0.4986
<i>Penicillium sp.</i>				
8	6	2.190	0	0.0076
9	14	2.261	0.063	0.332
10	20	3.764	1.574	0.265
11	25	3.764	1.574	lost
12	35	†3.820	1.630	0.4515
13	56	†4.101	1.911	0.5570

* Transformation of tannic acid complete.

† Considerable tannic acid still left in culture solution.

fication occurred. According to Malfitano³¹ the secretion of the enzymes from *Aspergillus* occurs just after fructification, and it is possible to conclude that the lack of transformation is explainable by non-secretion of the enzyme by the submersed growth. VanTieghem,³² however, found that transformation was effected when the growth was submersed; and some experiments of the writer subsequently confirm the fermentation by submersed growth. The non-transformation is probably due to the inhibiting action of the sugar on the secretion of the enzyme tannase, but not on the formation of the enzyme, for subsequent experiments have shown that with 2 per cent tannic acid and 10 per cent sugar the enzyme is formed. The inhibition of enzyme secretion by the presence of organic nutrients has been observed by Puriewitsch.³³

³¹ G. Malfitano: La proteolyze chez l'*Aspergillus niger*, *Ann. d. l'Ind. Pasteur*, xiv, pp. 60-81, 1900.

³² *Loc. cit.*

³³ K. Puriewitsch: Ueber die Spaltung der Glycoside durch die Schimmelpilze, *Ber. d. deutsch. bot. Gesellsch.*, xvi, pp. 368-377, 1898.

The transformation of salicin or arbutin was inhibited in the presence of certain amounts of glucose, cane sugar or starch. Likewise Katz²⁴ found that *Penicillium glaucum* did not secrete the enzymediastase when, along with 0.25 per cent soluble starch, either 2 per cent glucose or 15 per cent cane sugar was offered. *Aspergillus niger* was not influenced noticeably in the secretion of the tannase by the presence of the 10 per cent cane sugar, while the *Penicillium sp.* was markedly influenced.

Another noteworthy fact in regard to the *Penicillium sp.* is the small increase of gallic acid between the twentieth and thirty-fifth day. It seems that the enzyme secreted was so very little, or of such feeble activity or destroyed, that a very limited transformation only was effected. Even at the end of fifty-six days the tannic acid was not entirely converted, and the increase of gallic acid after the end of thirty-five days was relatively small.

Influence of concentration of sugar on growth. For an explanation of the protective action of 10 per cent sugar the following table is suggestive. Each result is the average of sixteen cultures of *Aspergillus niger*.

TABLE VIII.

CULTURE NO.	COMPOSITION OF NUTRIENT SOLUTION	SUGAR IN CULTURE SOLUTION	DRY WEIGHT OF FUNGUS
		per cent	grams
1	Check: 50 cc. sol. A + trace Fe ₂ Cl ₃ + 2.5 gms. sugar.....	5	0.779
2	50 cc. sol. A + trace Fe ₂ Cl ₃ + 8.55 gms. sugar.....	17.1	1.239
3	50 cc. sol. A + trace Fe ₂ Cl ₃ + 17.1 gms. sugar.....	34.2	1.513
4	50 cc. sol. A + trace Fe ₂ Cl ₃ + 25.65 gms. sugar.....	51.3	1.590
5	50 cc. sol. A + trace Fe ₂ Cl ₃ + 37.00 gms. sugar.....	74.0	1.308
6	50 cc. sol. A + trace Fe ₂ Cl ₃ + 42.50 gms. sugar.....	85.0	1.230

The table shows that the optimum sugar content is above 5 per cent. It is possible that the increased growth in the culture solution of over 10 per cent sugar is produced not by an assimilation

²⁴ J. Katz: Die regulatorische Bildung von Diastase durch Pilze, *Jahrb. f. wiss. Bot.*, xxxi, pp. 599-618, 1898.

lation of more sugar but by a stimulus, the result of high concentration. The table is offered here, however, only to indicate that with the nutrient solution used 5 per cent sugar is not sufficient for greatest growth. It throws light also upon the failure of the 5 per cent sugar to protect the gallic acid in the previous experiment.

VII. ELECTION OF ORGANIC SUBSTANCES.

Historical. VanTieghem²⁵ stated that the glucose formed as a result of tannic acid fermentation was utilized and the gallic acid left behind. Pasteur demonstrated that *Penicillium glaucum* exhibited an election of the dextro-tartaric acid when both the dextro- and laevo-tartaric acids were present. Duclaux's²⁶ observations revealed the fact that when *Aspergillus niger* was offered salts of butyric and acetic acid in a mixture, it first used the latter and then the butyric acid. Furthermore, he proved that this was not due to the better nutrient value of acetic acid, for when the acetate was offered with the tartrate (an especially good nutrient) the acetate was utilized more rapidly. The election then was not merely a matter of relative food value.

Pfeffer²⁷ found that under certain conditions the use of glycerin by fungi may be protected by dextrose and even better by peptone, and he showed also that the relative concentration of each had an effect upon the election.

Puriewitsch²⁸ found with two organisms an election with respect to the products of amygdalin. With this substance as the source of carbon it was first transformed; then the dextrose and lastly the benzaldehyde was used. He found further that salicin was not transformed in the presence of six times its quantity of dextrose, twelve times its quantity of saccharose or fourteen to sixteen times its quantity of starch.

Election of cane sugar. In order to determine definitely whether or not *Aspergillus niger* and *Penicillium sp.* elect cane sugar, when it is offered together with gallic acid, two series of cultures were made. Solution B was used, to which was added in the one case the gallic acid and 10 per cent of cane sugar, the cultures being made in Erlenmeyer flasks as before. In a similar manner cultures were made in which the gallic acid alone was offered as the source of carbon. Sterilization and inoculation were made by the usual

²⁵ *Loc. cit.*

²⁶ E. DuClaux: Sur la nutrition intracellulaire, *Ann. de l'Inst. Pasteur*, iii, pp. 97-112, 1889.

²⁷ W. Pfeffer: Ueber Election organischer Nährstoffe, *Jahrb. f. wiss. Bot.*, xxviii, pp. 205-268, 1895.

²⁸ *Loc. cit.*

methods, and the cultures incubated at 28° C. The results obtained are included in table IX.

TABLE IX.

ORGANISM	DURATION days	GALLIC ACID IN CULTURE SOLUTION grams	GALLIC ACID USED grams	DRY WEIGHT OF FUNGUS grams
SERIES I. <i>Solution B + 10 per cent sugar + gallic acid.</i>				
Check.....		2.837		
<i>Aspergillus niger</i>	7	2.837	none	0.3491
<i>Penicillium sp.</i>	7	2.837	none	0.1109
<i>Aspergillus niger</i>	10	2.837	none	0.4589
<i>Penicillium sp.</i>	10	2.837	none	0.3676
SERIES II. <i>Solution B + gallic acid.</i>				
Check.....		2.837		
<i>Aspergillus niger</i>	7	2.429	0.337	0.1000
<i>Penicillium sp.</i>	7	2.837	not detected	0.010
<i>Aspergillus niger</i>	10	1.474	1.363	0.3434
<i>Penicillium sp.</i>	10	2.557	0.280	0.108

A glance at the table reveals the fact that both *Penicillium sp.* and *Aspergillus niger* elect cane sugar and leave behind in the culture solution the gallic acid. Cane sugar is therefore proven conclusively to protect the gallic acid. It is of interest to note that the addition of the sugar permits of a more rapid and more extensive growth during the ten-day period.

VIII. INFLUENCE OF AERATION.

Limiting supply of oxygen. VanTieghem³⁹ stated that under aerobic conditions the tannic acid was utilized directly, and that the small amount of gallic acid formed was also assimilated. The preceding experiments and others of the writer, not here mentioned, show that transformation of the tannic acid occurs even when all the growth is on the surface. However, if sugar is not offered with the tannic acid, the increased growth may, as shown, be at the expense of the gallic acid formed. If the tannic acid is

³⁹ *Loc. cit.*

offered as the only source of carbon, the yield of gallic acid is obviously dependent upon the amount of growth. Van Tieghem⁴⁰ found that 0.022 gram's weight of mycelium transformed 48.3 grams of tannic acid in ten days at 35°. The growth is greatly diminished by the absence of oxygen, consequently the oxygen supply is a factor influencing the yield of gallic acid. In order to compare the yield under aerobic and anaerobic conditions, another experiment was performed. Solution B was used, to which was added tannic acid. Into each of six Erlenmeyers of 150 cc. capacity were placed 50 cc. of the solution. Four of the flasks were plugged with cotton; while the other two were fitted with perforated rubber stoppers. After sterilization the perforations were plugged with pieces of glass rod and these, together with two of the flasks plugged with cotton, were inoculated with *Aspergillus niger*. The flasks fitted with the rubber stoppers contained approximately 100 cc. of air and therefore about 20 cc. of oxygen. The cultures were incubated at 31° and at the end of twenty-eight days and forty days analyses were made. The results follow in table X.

TABLE X.

CULTURE SOLUTION	DURATION	TANNIC ACID IN CULTURE SOLUTION	LOSS OF TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS OR GAIN IN GALLIC ACID	DRY WEIGHT OF FUNGUS
	days		grams	grams	grams	grams
Anaerobic (limited).....	40	0	1.603	3.286	+1.186	0.156
Aerobic.....	28	0	1.603	0.898	-1.202	0.634
Check.....		1.603		2.100		

It is at once evident that the inhibition of growth due to deficiency of oxygen is favorable to a good yield of gallic acid. No doubt if the culture in limited oxygen supply had been analyzed sooner a greater yield of gallic acid could have been obtained. With respect to the condition in aerobic cultures it may be stated that cultures, identical with those above, showed on the fourth day a gain of 0.484 gram of gallic acid, though only half of the tannic acid had been transformed, and the weight of the mycelium produced was 0.0314 gram.

Comparison of methods. In order to determine more definitely the yield of the gallic acid under conditions in which the supply of

⁴⁰ *Loc. cit.*

oxygen varied, as well as to compare the yields under these conditions with that obtained when the most favorable conditions were offered, as by the addition of sugar, the following experiment was made:

SERIES I. Solution B + tannic acid, flask plugged with cotton and aerobic conditions maintained.

SERIES II. Solution B + tannic acid + 10 per cent cane sugar, otherwise like the above.

SERIES III. Solution B + tannic acid, flasks stoppered with rubber stoppers and containing therefore only 75 cc. of air or approximately 15 cc. of oxygen.

SERIES IV. Solution B. + tannic acid, flasks stoppered with perforated rubber stoppers, fitted with glass and rubber tubing and clamps. The air was replaced by passing a stream of nitrogen (oxygen-free air) through the flasks for a period of five minutes after inoculation had been made.

All the inoculations were made with spores of *Aspergillus niger*, according to the method described by Hasselbring.^a The temperature of incubation varied from 30°-35°. Erlenmeyer flasks of 125 cc. capacity were employed. The results obtained are included in table XI.

TABLE XI.
Aspergillus niger. Duration, ten days.

SERIES	ATMOSPHERE OF GROWTH CHAMBER	TANNIC ACID IN CULTURE SOLUTION	LOSS OF TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	GAIN IN GALLIC ACID	DRY WEIGHT OF FUNGUS
		grams	grams	grams	grams	gram
Check		4.433		3.089		
I	Unlimited air supply	0.409	4.024	5.618	2.529	0.3166
II	Unlimited air supply	0.683	3.760	6.515	3.426	0.3341
III	75 cc. air	1.023	3.410	6.151	3.062	0.0114
IV	Nitrogen	1.707	2.726	5.420	2.331	0.0013

In comparing the rate of tannic acid transformation under the different conditions it is found that in the order of rapidity of transformation they are series I to series IV. The yield of gallic acid was greatest in series II, the addition of cane sugar in this case protecting the gallic acid; somewhat less in series III; still less in series I, where the mycelium was abundant, despite the fact that it led in the amount of tannic acid transformed; and last in series IV. It is noteworthy that the amount of mycelium pro-

^a *Loc. cit.*

duced in this last was only 1.3 mgm., yet sufficient of the enzyme was liberated to transform a quantity of tannic acid more than 2000 times the weight of the mycelium produced.

From an economic standpoint the method of series I is wasteful of gallic acid, as the organism utilizes much of this substance in its metabolism. Series II has an advantage over series III, and series III over series IV, only in the rapidity of the transformation. The small amounts of growth in series III and IV require such a slight amount of gallic acid in their metabolism that the yield of gallic acid in those series would finally be practically equal to that obtained in series II, to the cultures of which sugar had been added. This fact is borne out by the larger amounts of tannic acid left in the culture solutions of series II and series III.

IX. SUMMARY.

1. Tannic acid is toxic to a large number of fungi at relatively low concentrations.
2. *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium sp.*
3. The fermentation was found to be more rapid in the gall nut infusion than in the synthetic solution in which tannic acid was the only source of carbon. The presence of other organic compounds in the gall nut infusion protected to a certain extent the gallic acid.
4. The addition of 5 per cent sugar did not protect the gallic acid but simply increased the growth. The addition of 10 per cent sugar protected the gallic acid entirely.
5. When gallic acid and cane sugar to the extent of 5.5 per cent and 10 per cent, respectively, were offered together, the cane sugar was elected and the gallic acid left in the culture solution.
6. Fermentation can take place under anaerobic conditions, and 1 mgm. of mycelium is sufficient to effect the transformation of 2.706 grams of tannic acid in ten days.
7. In an approximately 15 per cent solution of tannic acid, fermentation was most rapid when the tannic acid alone served as the source of carbon, and when aerobic conditions were maintained, yet the method of fermentation is wasteful from the standpoint of an economical yield of gallic acid.

8. The economical methods are (a) those in which growth occurs under aerobic conditions and the tannic acid is supplemented by cane sugar; or (b) those in which, with tannic acid alone, the supply of oxygen is limited to a small amount.

9. The presence of 10 per cent cane sugar does not inhibit the secretion of the enzyme tannase by *Aspergillus niger*, but it does seem to inhibit to some extent the secretion of the tannase by *Penicillium sp.*

10. The enzyme is secreted into the culture solution by submerged mycelium as well as by surface growth. There is no evidence that tannic acid is used directly; but the evidence seems to indicate that tannic acid is first transformed into gallic acid and the gallic acid then utilized.

APPENDIX.

Investigators previously occupied with tannic acid fermentation usually employed *Aspergillus niger* together with a species of *Penicillium* which they have designated *Penicillium glaucum*. As has been pointed out by Thom⁴² the name *Penicillium glaucum* has in the past been applied to so many different species that the only idea conveyed by its use is a general concept of the genus. VanTieghem⁴³ applied the name to denote the species of *Penicillium* which he isolated from gall nuts, and it is probable that other students of tannic acid fermentation used the same organism.

In the work of the writer a trial of a number of organisms was made and the *Penicillium* employed was secured from a culture labeled *Penicillium olivaceum*. That identification was incorrect, and then it was believed that the organism might be the one which develops on the gall nuts, but this also proved erroneous, as is indicated subsequently.

In attempting to determine the *Penicillium sp.* used in these experiments it was found that the organism did not correspond to any of the species described by Thom.⁴⁴ Instead, however, of withholding publication of these investigations until the organism should be definitely determined, it was thought best to present here a brief description and certain cultural characteristics of the organism. It is, furthermore, the intention of the writer to make a study of the relation of the various species of *Penicillium* to tannic acid fermentation, and it is hoped by that time to have determined definitely the two species of *Penicillium* which are now known to develop in a 10 per cent solution of tannic acid.

The *Penicillium sp.* used in these experiments possesses only a single whorl of unbranched conidia-bearing cells (sterigmata), and might therefore

⁴² Charles Thom: Cultural Studies of *Penicillium*, Bureau of Animal Industry, Bulletin 118, pp. 107, 1910.

⁴³ *Loc. cit.*

⁴⁴ *Loc. cit.*

be grouped with the genus *Citromyces* as founded by Wehmer; but Thom⁴⁵ does not consider this a valid basis for differentiation of genera, and prefers to include this form of conidiophore under the genus *Penicillium*. This latter concept is here followed.

Description of organism. Colonies on 15 per cent gelatin are, when young, of a faint green color which changes to an otter brown. On 15 per cent gelatin + 3 per cent sugar the olive green changes to ashy gray and then to greenish black. On bean agar the color is at first bluish green, and changes to dark olive green, and finally to a grayish color. The surface is velvety. The conidiophores arise vertically from the substratum and in length vary from 100μ to 700μ . The fructification averages 90μ in length (it may be 200μ), and its width is approximately 15μ . The conidiiferous cells average 10μ in length, the conidia are spherical, and 3μ in diameter. A single whorl of simple conidia-bearing cells only is present as is represented by figures 1 and 2.



FIG 1.

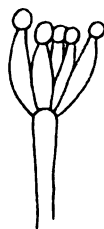


FIG 2.

Conidial fructification showing simple conidiiferous cells ($\times 600$).

Cultural character. At room temperature 15 per cent gelatin is liquified in six days with the production of a strong ammoniacal odor; in the presence of 3 per cent sugar the total liquefaction is retarded a day or more. The presence of bean juice still longer delays the liquefaction of the gelatin. On 15 per cent gelatin + 3 per cent sugar, the lower surface is colored yellowish to reddish brown, when grown in solution B + 10 per cent cane sugar the lower surface of the mycelial felt may be of a salmon color. Fruiting on such a solution at room temperature usually requires eight days. This organism possesses the ability to ferment tannic acid and with 10 per cent tannic acid in solution B at a temperature of 30°C ., gallic acid may be precipitated in about seven days.

Gall nut Penicillium. Only one other species of *Penicillium* has so far been found to grow on 10 per cent tannic acid, and this is the one isolated from the gall nuts. It grows more slowly in 10 per cent tannic acid. In the presence of sugar it produces an intensely red color in the substratum and is a slow liquefier of gelatin. It differs from the other also in possessing more than one whorl of conidiiferous cells, and has other distinguishing features. It appears to be *Penicillium rugulosum*.

⁴⁵ *Loc. cit.*

TANNIC ACID FERMENTATION. II.

EFFECT OF NUTRITION ON THE PRODUCTION OF THE ENZYME TANNASE.

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I. PREFACE.

In an investigation upon tannic acid fermentation reported in the previous paper, it was found that when cane sugar and tannic acid are offered simultaneously to either *Aspergillus niger* or *Penicillium sp.*, the sugar is utilized as the source of carbon while the tannic acid is fermented, gallic acid resulting. Some of the results indicated that the rate of fermentation was influenced by the concentration of the sugar. It was deemed important, therefore, to determine if varying the relative amounts of tannic acid and sugar in the nutrient solution has an influence upon the amount of the enzyme tannase produced in the fungus thus grown. Since tannic acid is probably not commonly utilized in nature by *Aspergillus niger* and *Penicillium sp.* as a source of carbon, experiments were also made to determine if the enzyme is produced when the fungus is cultivated on nutrient solution lacking tannic acid.

The writer wishes to acknowledge his indebtedness to Prof. B. M. Duggar for assistance received during the course of the investigation.

II. INTRODUCTION.

Regulatory production of enzymes. Historical. A number of investigations have been made on the regulatory formation of the enzymes, but for the most part conclusive investigations are lacking. Experimenting with two species of bacillus, Brunton and MacFayden¹ found that when culti-

¹ T. Lauder Brunton and A. MacFayden: The Ferment Action of Bacteria, *Proc. Roy. Soc.*, xlv, B, pp. 543-553, 1899.

vated on starch paste these developed the enzyme diastase; but if the starch were replaced by meat extract no diastase was formed. Fermi² found that of ten bacterial organisms, which developed the tryptic ferment in the presence of peptones or albumen, none developed the enzyme on a sugar-containing nutrient solution. Wortman's³ observations established the fact that the addition of tartaric acid prevented the formation of diastase in bacteria which inhabited decaying potatoes. Fermi and Montesano's⁴ investigations indicated that the presence of sugar is not absolutely necessary for the formation of the enzyme invertase. Various other investigators have studied the influence of nutrition on the formation of enzymes in bacteria.

Dubourg⁵ stated that a yeast which did not normally possess the inverting enzyme was capable of developing it by proper cultivation. As a culture solution in the latter case, yeast water was used, to which was added 5 per cent cane sugar and 5 per cent grape sugar. The yeast, after cultivation, was thoroughly washed and then transferred to a cane sugar solution. In the latter inversion occurred. The form of yeast was not an identified strain. He reported that he was also able to develop the enzyme which fermented galactose by similar methods.

Klöcker,⁶ employing the methods of Dubourg, was unable to develop invertase in *Saccharomyces apiculatus* or maltase in *Saccharomyces maris-anus* which organisms do not normally possess these enzymes. The ability to form specific enzymes, according to Klöcker, is therefore a constant character of the yeast organism.

Recently Harden and Norris⁷ "have trained" the yeast *Saccharomyces Carlsberg I* to ferment galactose by cultivating the yeast on hydrolyzed lactose in yeast water to which was added 0.15 per cent of monobasic potassium phosphate. Normally galactose is not fermentable by the above mentioned yeast. According to Kohl⁸ the chemical nature of the solution and the aeration of the culture influence the amount or activity of the enzyme formed in the yeast organism, while the temperature of storage of the yeast also markedly affects the enzyme content.

² Claudio Fermi: Weitere Untersuchungen über tryptischen Enzyme der Mikroorganismen, *Centralbl. f. Bact.*, x, pp. 401-408, 1891.

³ J. Wortman: Untersuchungen über das diastatische Ferment der Bacterien, *Zeitschr. f. physiol. Chem.*, vi, p. 287, 1882.

⁴ C. Fermi and C. Montesano: Die von den Mikroben bedingte Inversion des Rohrzuckers, *Centralbl. f. Bact.*, i, Abt. II, pp. 482-87, 542-56.

⁵ E. Dubourg: De la fermentation des saccharides, *Compt. rend. de l'Acad. des Sci.*, cxxviii, pp. 440-42.

⁶ Alb. Klöcker: Ist die Enzymebildung bei der Alcoholgärungspilzen ein verwertbares Artmerkmal?, *Centralbl. f. Bact.*, vi, Abt. II, pp. 241-45, 1900.

⁷ A. Harden and R. V. Norris: The Fermentation of Galactose by Yeast and Yeast Juice, *Proc. Roy. Soc.*, lxxxii, B, pp. 645-49, 1910.

⁸ F. G. Kohl: *Die Hefpilze*, 1908, pp. 79-81.

Büsgen⁹ showed that *Aspergillus oryzae* on bouillon, as well as in a sugar-containing solution, formed the enzyme diastase. According to Pfeffer,¹⁰ *Penicillium glaucum* did not secrete diastase in the presence of 10 per cent sugar and, even when only 1.5 per cent sugar was present, the starch was only slightly attacked. *Aspergillus niger* behaved differently, producing diastase even in the presence of 30 per cent cane sugar. Employing a nutrient solution containing 0.25 per cent of soluble starch Katz¹¹ found that starch was saccharified by *Penicillium glaucum*. The addition of 2 per cent grape sugar or 1.5 per cent cane sugar prevented the formation of the diastase. An addition of 1.5 per cent cane sugar depressed the formation of the diastase, while an addition of 0.05 per cent had no effect. Lactose and maltose in a 3 per cent concentration decreased the rate of starch transformation, while a 10 per cent concentration still further depressed the formation of diastase. A 4 per cent addition of erythrodextrin had no effect whatsoever in protecting the starch. Neither did a 10 per cent addition of quinic acid, 4 per cent glycerin or 2 per cent potassium tartrate have any effect upon the secretion of the diastase. The addition of peptone to the solution increased the secretion of the diastase. With *Aspergillus niger* the growth on starch nutrient solution was slow, and five days were required for the transformation of the starch. The addition of 1.5 per cent cane sugar decreased the time to two days, 15 per cent sugar increased the time of transformation by one day and 30 per cent sugar increased the time by two days. *Bacterium megatherium* behaved much the same as *Penicillium glaucum*.

Dox¹² has shown that the carbohydrate-splitting enzymes, amylase, inulase, raffinase, sucrase, maltase and lactase are formed in *Penicillium camemberti*, regardless of the carbohydrate which has served as the source of carbon in the nutrient solution. The amount of the particular enzyme could be increased, however, by cultivating the organism on the corresponding carbohydrate. Likewise, other enzymes are formed independently of the presence in the nutrient solution of the corresponding substance on which the enzyme acts.

According to Went,¹³ the ten enzymes which he investigated in *Monillia sitophila* could be divided into three groups according to the influence of nutrition on their formation. The first group includes those which are formed in slight amounts regardless of the nutrition, the second group in-

⁹ M. Büsgen: *Aspergillus oryzae*, *Ber. d. deutsch. bot. Gesellsch.*, iii, pp. 66-77, 1885.

¹⁰ Quoted from R. Green: *The Soluble Ferments and Fermentation*, p. 32.

¹¹ J. Katz: Die regulatorische Bildung von Diastase durch Pilze, *Jahrb. f. wiss. Bot.*, xxxi, pp. 599-618, 1898.

¹² A. W. Dox: The intracellular Enzymes of *Penicillium* and *Aspergillus*, U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120, 70 pp., 1910.

¹³ F. C. Went: Ueber den Einfluss der Nahrung auf die Enzyymbildung durch *Monillia Sitophila* (Mont.), Sacc., *Jahrb. f. wiss. Bot.*, xxxvi, pp. 611-664, 1901.

cludes the enzymes which are formed only under several different forms of nutrition, while the third group includes such enzymes as are formed only when the substance on which the enzyme acts is present in the culture solution.

Butkewitsch¹⁴ also has shown that nutrition has an influence upon the secretion of the gelatin-dissolving enzyme by *Aspergillus* and *Penicillium*.

III. METHODS OF EXPERIMENTATION.

In all of the experiments upon the influence of nutrition on the production of the enzyme the organisms were cultivated in a synthetic nutrient solution, the inorganic composition being that of Czapek's¹⁵ formula. Throughout this paper the solution has been designated for convenience solution B and is as follows:

Magnesium sulphate (cryst.).....	0.5 gram
Dibasic potassium phosphate.....	1.0 gram
Potassium chloride.....	0.5 gram
Sodium nitrate.....	2.0 grams
Distilled water.....	1000 cc.

All cultures were grown at a temperature of 28° or 30°. The fungus felt, when formed and before spore production, was removed from the nutrient solution, as it has been shown by Malfitano¹⁶ that the most abundant secretion of enzymes into the culture solution takes place just after spore formation. The felt after removal was treated according to Albert and Buchner's method for the preparation of "Acetondauerhefe," as described by Dox,¹⁷ though in the method here employed the felt was not run through a bashing machine. After the mycelium was dry, it was pulverized in a mortar and then placed in a vial until its enzymatic activity was to be determined.

For determining the presence of the enzyme or the relative

¹⁴ W. Butkewitsch: Umwandlung der Eiweisstoffe durch die niederen Pilze im Zusammenhange mit einer Bedingungen ihrer Entwicklung, *Jahrb. f. wiss. Bot.*, xxxviii, pp. 147-240, 1903.

¹⁵ Quoted from A. W. Dox: The Intracellular Enzymes of *Penicillium* and *Aspergillus*, U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120, 70 pp., 1910.

¹⁶ G. Malfitano: La proteolyse chez l'*Aspergillus niger*, *Ann. de l'Inst. Pasteur*, xiv, pp. 60-81, 1900.

¹⁷ A. W. Dox: The Intracellular Enzymes of *Penicillium* and *Aspergillus*, *loc. cit.*

amount of it present, the procedure was as follows: Either a 0.5 per cent, a 0.75 per cent or a 1.0 per cent solution of tannic acid was employed. To the flasks containing the solution were then added equal weights of the pulverized mycelium, the enzymatic activity of which was to be determined. There was also added, as an antiseptic, 2 per cent toluene. The flasks containing the solution and mycelium powder, being tightly stoppered, were then incubated for a definite period and then the solution analyzed for gallic acid according to Jean's¹⁸ method. The relative increase in the gallic acid is taken as a measure of the amount of tannase present.

IV. INFLUENCE OF CONCENTRATION OF SUGAR AND TANNIC ACID ON PRODUCTION OF TANNASE.

Influence of concentration of tannic acid on the amount of enzyme produced. It was found in certain experiments,¹⁹ in which 10 per cent sugar plus tannic acid had been added to the nutrient solution, that the addition of sugar could not prevent the secretion of the enzyme tannase by *Aspergillus niger*, but the secretion by *Penicillium sp.* was markedly decreased. In order to determine the minimum concentration of tannic acid which would stimulate the formation of the enzyme tannase and also what influence the concentration of tannic acid would have upon the amount of tannase produced within the organism, the following experiment was made: Solution B was used, to which was added 10 per cent cane sugar. Liter flasks were employed, and to each were added 500 cc. of the solution. The flasks were plugged and sterilized, and when cool the tannic acid was added to each in varying amounts, as is indicated in the table. For inoculation the method described by Hasselbring²⁰ was employed, though 1 cc. of the spore-containing water was used instead of a single drop. These cultures were incubated at 28°C., and the felt was then removed and treated according to the method described. In determining the enzymatic activity of the mycelium powder 0.3

¹⁸ F. Jean: Die Bestimmung des Tannins und der Gallussäure, *Chem. Centralbl.*, 1900, pp. 1107-08.

¹⁹ L. Knudson: Tannic acid Fermentation I, this *Journal*, xiv, p. 159, 1913.

²⁰ H. H. Hasselbring: Carbon Assimilation of *Penicillium*, *Bot. Gazette*, xlv, pp. 176-193, 1908.

gram of the dried powder was added to a flask containing the tannic acid solution. Each flask contained 75 cc. of an approximately 0.9 per cent solution to which was added, as an antiseptic, 1 cc. of toluene. The results after one week's incubation at 34° are given in table I. The solution contained at the beginning of incubation 0.555 gram tannic acid and 0.327 gram gallic acid.

TABLE I.

Effect of concentration of tannic acid on production of enzyme tannase; using nutrient solution B + 10 per cent sugar + tannic acid. Period of incubation, 7 days.*

	AMOUNT OF TANNIC ACID ADDED		GAIN IN GALLIC ACID			AMOUNT OF TANNIC ACID ADDED		GAIN IN GALLIC ACID
	per cent	gram				per cent	gram	
<i>Aspergillus niger</i>	0.00	0	<i>Penicillium</i> sp.	}	0.01	0	}	
	0.01	0			0.10	0.058		
	0.10	0.059			0.40	0.05		
	0.80	0.223			0.80	0.065		
	1.00	0.272			1.00	0.108		
	2.00	0.388			2.00	0.159		
	4.00	0.515			4.00	0.293		
	10.00	0.515						
	17.00	0.525						
		No sugar						

* The actual concentration of each culture was approximately only two-thirds of the figures given, the other one-third consisting of gallic acid.

It is evident from the preceding table that there is a regulatory formation of the enzyme. There is a progressive increase in the amount of tannase with increase of tannic acid in the culture solution. It is noteworthy that no tannase was produced when growth took place in a nutrient solution which lacked tannic acid. It is somewhat remarkable that the formation of enzyme could be stimulated by 0.1 per cent of tannic acid (actually about 0.066 per cent) when there was present at the same time cane sugar in an amount more than one hundred times as great as the tannic acid. The stimulation by this small amount of tannic acid is even more surprising when previous experiments²¹ are recalled in which the gallic acid formed from the tannic acid was protected by the cane sugar, no determinable amount of the gallic acid being assimilated.

²¹ L. Knudson: *loc. cit.*

Why the increase in concentration of tannic acid should increase the amount of tannase is difficult to explain. The amount removed by the organism from the solution is undeterminable with the present method of analysis.

Is the stimulation produced within the cell, or is it caused by contact of the tannic acid with the plasma membrane? Tannic acid precipitates albuminous substances, and it might be possible that it reacts in this manner with the plasma membrane and this precipitation might be the stimulus for the production of the enzyme. This explanation would not, however, include the stimulation to production of the tannase by gallic acid.

A suggestive explanation for the increase of the tannase with increased concentration of the tannic acid is afforded by the work of Katz.²² In his experiments Katz found that diastase could be precipitated by tannic acid and rendered inactive, though when freed from the tannic acid by washing with alcohol it becomes active. Bearing in mind this precipitation by tannic acid and working on the hypothesis that if the diastase formed and secreted into the culture solution were removed from solution more diastase would be formed, he added tannic acid to the culture solution, assuming that in this way there should be an increase in the quantity of the diastase formed. In his experiment Katz actually found that the total quantity of enzyme formed (that secreted into the nutrient solution and that present in the fungus) was greater in the culture which contained 0.5 per cent tannic acid than in the control, the ratio of the diastatic activity being 143 to 100. The results seem to confirm his hypothesis, but there are a number of factors which suggest another explanation of the results obtained. In some previous experiments²³ of the writer it is noted that in the presence of 10 per cent sugar the tannic acid was fermented, and in table I, here reported, it is clear that even in the presence of only 0.1 per cent tannic acid the enzyme tannase is formed and in all probability secreted. The tannic acid of the culture solution would, therefore, be fermented and rendered inactive as regards its capacity to precipitate the diastase liberated, and this condition doubtless occurred in the experiment of Katz.

²² J. Katz: Die regulatorische Bildung von Diastase durch Pilze, *Jahrb. f. wiss. Bot.*, xxxi, pp. 599-618, 1898.

²³ *Loc. cit.*

TABLE III.

Aspergillus niger.

Effect of concentration of sugar on production of tannase; using solution B + 2 per cent tannic acid + cane sugar. Period of incubation, ninety hours

AMOUNT OF CANE SUGAR ADDED	INCREASE OF GALLIC ACID	AMOUNT OF CANE SUGAR ADDED	INCREASE OF GALLIC ACID
<i>per cent</i>	<i>gram</i>	<i>per cent</i>	<i>gram</i>
0	0.224	+12	0.166
+1	0.206	+16	0.134
+2	0.206	+24	0.130
+8	0.179		

sugar, the tannic acid being maintained at a constant figure, there is a decrease in the amount of the enzyme produced.

In order to verify the above results another experiment was made along substantially the same lines with *Aspergillus niger*. To test the tannase activity of the dry mycelium 100 cc. of a 0.5 per cent tannic acid solution were used to which was added 1 per cent toluene. In each case 0.05 gram of the powdered mycelium was employed. The cultures were made in duplicate and the determinations likewise. One set was incubated at 24°C. for four days and the second set at the same temperature for six days before analyses were made for gallic acid. In the following table there is given the composition of the nutrient solutions used and the increase in the gallic acid resulting, which increase of course is a measure of the tannase activity of the various cultures.

TABLE IV.

Aspergillus niger.

Effect of concentration of sugar on production of tannase; using solution B + 2 per cent tannic acid + cane sugar.

AMOUNT OF SUGAR ADDED	DRY WEIGHT OF FUNGUS AVERAGE OF 2	INCREASE IN GAL- LIC ACID; 4 DAYS INCUBATION	INCREASE IN GAL- LIC ACID; 6 DAYS INCUBATION	AVERAGE INCREASE
<i>per cent</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>
32	0.158	0.179	0.179	0.179
16	0.083	0.270	0.278	0.274
12	0.074	0.233	0.270	0.251
8	0.101	0.270	0.278	0.274
4	0.093	0.270		0.270
2	0.065	0.270	0.296	0.283
0.5	0.074	0.303	0.332	0.317
0	0.035	0.303	0.368	0.335

The results confirm the evidence of the preceding experiment, though the differences due to sugar concentration are not so marked. This may have been due to the longer period of incubation.

V. INFLUENCE OF NUTRITION.

Since Dox has found in *Penicillium camemberti* that the various enzymes are produced irrespective of the nutrition of the fungus, an experiment was made to determine definitely if the enzyme tannase could be produced in two *Penicillium* species when tannic acid is withheld from the culture solution. *Penicillium sp.*, which I have previously described,²⁵ and *Penicillium rugulosum* were employed in the experiment. They were cultivated in 100 cc. of a nutrient solution which was composed on the one hand of solution B + 5 per cent sugar and, on the other, solution B + 5 per cent sugar + 2 per cent tannic acid. The mycelial felts, as before, were removed just before spore formation, treated as previously described and pulverized. For determining the presence of the enzyme tannase 100 cc. of a 1 per cent tannic acid solution were used to which was added 2 per cent toluene as an antiseptic and 0.1 gram of the pulverized mycelium. The solutions were incubated for twenty-eight days at a temperature of 33°. They were then analyzed for increase in gallic acid. All cultures and determinations were made in triplicate. As is evident from the table tannase was formed only in the presence of tannic acid.

TABLE V.

ORGANISM	COMPOSITION OF SOLUTION	GALLIC ACID PRESENT	GAIN
<i>Penicillium sp.</i>	Solution B + 5 per cent sugar	0.287	0
<i>Penicillium rugulosum.</i>	Solution B + 5 per cent sugar	0.287	0
<i>Penicillium sp.</i>	Solution B + 5 per cent sugar + 2 per cent tannic acid	0.822	0.535
<i>Penicillium rugulosum.</i>	Solution B + 5 per cent sugar + 2 per cent tannic acid	0.822	0.535
Check.....		0.287	0

Experiments were next made to determine the influence of replacing the sugar by other compounds and of adding to the nutrient solution various reagents. For this experiment solution

²⁵ *Loc. cit.*

A²⁶ was used, and as culture vessels liter flasks were employed. Into each flask were placed 365 cc. of the solution, and to it were added the sugar and other reagent, or the sugar was omitted and some other carbon compound substituted for it. The solutions were sterilized, inoculated and incubated at a temperature of 28°C. The felt was removed just before spore production and treated in the manner previously described.

To determine the presence of tannase, 0.3 gram of the powdered, dried mycelium was introduced into 100 cc. of a 0.75 per cent solution of tannic acid, to which had been added as an antiseptic 2 cc. of chloroform. After incubation at 31° for one week, the solutions were analyzed for gallic acid. The results obtained are given in table VI.

TABLE VI.
Penicillium sp.

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID PRESENT	GALLIC ACID INCREASE
Solution A + 25 grams cane sugar.....	0.202	0
Solution A + 25 grams cane sugar 5 cc. $\frac{N}{10}$ HCl..	0.202	0
Solution A + 15 grams corn starch.....	0.208	0
Solution A + 25 grams glycerin.....	0.202	0
Solution A + 25 grams gallic acid.....	0.370	0.128
Solution A + 25 grams tannic acid.....	0.713	0.511

The control contained 0.202 gram gallic acid at the beginning and at the end of the incubation.

The gallic acid stimulated the formation of the enzyme only one-fourth as much as did the tannic acid. Slight acidity had no effect in stimulating the production of the enzyme. Glycerin and starch, both of which are relatively poor food compounds, were supplied, and if enzymes are stimulated to formation by conditions approaching starvation, as suggested by Wortman,²⁷ then the tannase should have developed; but the results were negative.

An experiment similar to that above was made with *Aspergillus niger*, 400 cc. of solution being used and the methods of experimentation the same as before. The results obtained are as follows:

²⁶ KH₂PO₄, 0.5 gram; KNO₃, 1 gram; MgSO₄, 0.25 gram; Distilled water, 100 cc.

²⁷ *Loc. cit.*

TABLE VII.

Aspergillus niger.

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID PRESENT	GALLIC ACID INCREASE
Solution A + 25 grams sugar.....	0.209	0
Solution A + 25 grams sugar + 5 cc. $\frac{N}{10}$ HCl.....	0.202	0
Solution A + 25 grams sugar + 5 grams gallic acid.....	0.202	0
Solution A + 25 grams sugar + 2 grams resorcin.	0.190	0
Solution A + 25 grams sugar + 1 grams hydro- quinone.....	0.202	0
Solution A + 25 grams sugar + 5 grams peptone.	0.202	0
Solution A + 25 grams sugar tannic acid.....	0.691	0.489

From the above table, it is evident that in *Aspergillus niger* there is a very marked regulatory formation of the enzyme. Pep-tone, which stimulates the secretion of diastase, according to Katz²⁸ has no influence in stimulating the formation of tannase. Gallic acid of the strength used had no effect, which result is surprising in view of the results indicated in table VI and of the further fact that Pottevin²⁹ found the enzyme tannase to be formed in *Aspergillus niger* when the organism is cultivated in Raulin's solution with the sugar replaced by gallic acid. Since pyrogallol is a decomposition product of tannic acid, it was thought that perhaps it might stimulate the formation of the enzyme, but the amounts used proved toxic in this particular experiment. Resorcin and hydroquinone were both employed because of their constitutional similarity to pyrogallol, both being dihydroxybenzenes, while the pyrogallol is a trihydroxybenzene. Negative results, however, were obtained.

In a similar manner experiments were made in which 10 per cent cane sugar plus various other substances were added to solution A. For the experiment 500 cc. of the solution were used. In determining the presence of tannase, 0.2 gram of the powdered mycelium was added to 75 cc. of a 0.9 per cent tannic acid solution to which had been added 2 per cent toluene as an antiseptic. The results are given in table VIII.

²⁸ *Loc. cit.*²⁹ H. Pottevin: La tannase. Diastase dédoublant l'acide gallotanique, *Compt. rend. de l'Acad. des Sci.*, cxxxi, pp. 1215-17, 1901.

TABLE VIII.
Aspergillus niger.

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID INCREASE
Solution B + 10 per cent sugar + 35 mgm $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$. .	None
Solution B + 10 per cent sugar + 20 mgm salicylic acid.	None
Solution B + 10 per cent sugar + 1 gram gallic acid . . .	None
Solution B + 10 per cent sugar + 200 mgm. methyl salicylate	None
Solution B + 10 per cent sugar + 200 mgm. ethyl salicylate	None
Solution B + 10 per cent sugar + 50 mgm. methyl salicylate	None
Solution B + 10 per cent sugar + 50 mgm. ethyl salicylate	None

Pottevin³⁰ states that tannase has the property of hydrolyzing methyl and ethyl salicylate. In my cultures the methyl or ethyl salicylate did not incite the development of the enzyme. The amount of salicylate present, however, was small. These concentrations may have been too weak to stimulate the production of the enzyme but probably the hydrolysis of these two substances is due to another enzyme. Salicylic acid also was used because of its relation to gallic acid, salicylic acid being a monohydroxybenzoic acid, while gallic acid is a trihydroxybenzoic acid. The former was necessarily used at a very low concentration, and was without effect. The zinc sulphate was used at a concentration which is stimulating to the fungus growth, as shown by Richards,³¹ but no formation of the tannase resulted.

Since in some of the experiments tannase was not produced when the organism was grown in a solution with the carbon supplied as cane sugar and gallic acid, an experiment was made with the carbon supplied only as gallic acid. At the same time experiments were made to determine the influence of certain glucosides. Solution B was used, and 500 cc. of it placed in each of three liter flasks. These were plugged and sterilization made at 115° for ten minutes. To each of the three flasks was added gallic acid, amygdalin and salicin, respectively, and the culture solutions

³⁰ *Loc. cit.*

³¹ H. M. Richards: Die Beeinflussung des Wachstums einiger Pilze durch chemische Reize, *Jahrb. f. wiss. Bot.*, xxx, pp. 665-88.

then inoculated. The growth in gallic acid was rapid, but not heavy. In the solutions in which the glucosides were present, growth was at first very slow, but after the transformation of the glucosides had begun, growth was rapid. While it required eight days to develop the first felts in the two glucoside solutions, the second felts (after the removal of the first) developed in two days. The felts first formed were removed and treated according to the methods previously described, and the powdered mycelium then added to 100 cc. of a 0.90 per cent solution of tannic acid which contained also 2 per cent of toluene. After incubating two days at 40°C., the solutions were analyzed for gallic acid. The composition of the nutrient solutions and the gallic acid formed by the powdered mycelium are given in table IX.

TABLE IX.
Aspergillus niger.

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID PRESENT	GALLIC ACID INCREASE
Solution B 500 cc. + 10 grams gallic acid.....	0.546	0.219
Solution B 500 cc. + 10 grams salicin.....	0.327	0
Solution B 500 cc. + 2 grams amygdalin.....	0.327	0

The control contained 0.327 gram gallic acid and 0.555 gram tannic acid.

In preceding tables it has been seen that gallic acid at certain concentration in the presence of 10 per cent sugar does not stimulate the formation of the enzyme tannase. When gallic acid is present alone as the source of carbon, the enzyme is produced. If tannic acid is a glucoside, it might be expected that the presence of another glucoside would stimulate the production of some tannase, but such was not the case. Nevertheless, glucosides were transformed by the organism. While the gallic acid stimulates the formation of the enzyme tannase, it does not do so as effectively as the tannic acid.

VI. EFFECT OF CONCENTRATION OF GALLIC ACID ON PRODUCTION OF TANNASE.

Since the amount of tannase produced is regulated by the relative concentrations of sugar and tannic acid it was deemed important to determine if the amount of the tannase produced could be in-

creased by increasing the concentration of the gallic acid. In the experiment, the results of which are given in table X, the usual methods were followed. Erlenmeyer flasks of 250 cc. capacity were used and 100 cc. of the nutrient solution. For determining the tannase activity of the various cultures 0.05 gram of the dried mycelium was added to 50 cc. of 1 per cent tannic acid solution and the solutions incubated for forty-eight and eighty hours respectively at a temperature of 30°. The culture solutions were tested for their tannase content by precipitating any enzyme present with alcohol and collecting on a filter any precipitate. The test for the enzyme was made as before. Incubation was made at a temperature of 30° for ninety hours. The results in table X are the averages of two cultures.

TABLE X.

Aspergillus niger.

Effect of concentration of gallic acid on the production of tannase. Average period of incubation for mycelial powder 64 hours and for enzyme excreted 90 hours.

COMPOSITION OF NUTRIENT SOLUTION	WEIGHT OF FUNGUS. AV. OF 2 CULTURES	INCREASE IN GALLIC ACID		
		Mycelium Av. of 2	Enzyme excreted. Av. of 2	Total increase in gallic acid
	gram	gram		gram
Solution B + 10 per cent sugar + 0.1 per cent gallic acid.....	0.240	0	0	0
Solution B + 10 per cent sugar + 0.5 per cent gallic acid.....	0.442	0.024	0	0.024
Solution B + 10 per cent sugar + 1.0 per cent gallic acid.....	0.324	0.045	0	0.045
Solution B + 10 per cent sugar + 1.5 per cent gallic acid.....	0.280	0.046	0	0.046

An examination of the above table reveals the fact that the enzyme tannase is produced when the concentration of gallic acid is 0.5 per cent in the presence of 10 per cent sugar. At a concentration of 1.0 per cent gallic acid in the presence of 10 per cent sugar the amount of tannase produced is double that produced when only 0.5 per cent gallic acid is present. In the presence of

10 per cent sugar the gallic acid, so far as my previous results²² show, is not at all absorbed from the nutrient solution. Why the increase in gallic acid should increase the amount of tannase produced is therefore difficult to explain. The writer is not prepared at present to offer an explanation.

VII. SUMMARY.

1. There is a progressive increase of tannase in the two organisms *Aspergillus niger* and *Penicillium sp.* with increased concentration of tannic acid in Czapek's solution containing 10 per cent sugar.

2. In a full nutrient solution containing, as a source of carbon, 2 per cent tannic acid, the addition of cane sugar decreases the quantity of the tannase produced by the organism. The higher the concentration of sugar the lower the quantity of tannase produced.

3. *Aspergillus niger* produces more tannase or a more active tannase per unit weight than *Penicillium sp.*

4. The production of tannase in *Aspergillus niger*, *Penicillium sp.* and *Penicillium rugulosum* is stimulated by tannic acid and gallic acid, only, and the former is more effective than the latter.

5. There is a progressive increase of tannase in *Aspergillus niger* with increased concentration of gallic acid in a nutrient solution containing 10 per cent sugar.

VIII. DISCUSSION.

Dox²³ in his excellent paper makes the following statement: "There is no evidence that enzymes not normally formed by the organism in demonstrable quantities can be developed by special methods in nutrition. The influence of adding a particular substance to the medium is, therefore, not to develop an entirely new enzyme, but to stimulate the production of an existing enzyme, which is normally formed under all conditions." In the light of Dox's results it seems somewhat surprising that the enzyme tannase should be formed only under special nutrition. It may be as Dox²⁴

²² *Loc. cit.*

²³ A. W. Dox: The Intracellular Enzymes of *Penicillium* and *Aspergillus*, *loc. cit.*

²⁴ A. W. Dox: Enzyme Studies of Lower Fungi, *Plant World*, xv, pp. 40-43, 1912.

has stated concerning my work that the formation of the enzyme tannase is an exception to the general rule. The work of other investigators previously mentioned has indicated, however, that the production of certain enzymes in other organisms is governed entirely by the character of the nutrition.

Dox³⁵ has considered only the influence of external environment upon the formation of the enzymes. It is to be expected that protease, lipase, nuclease, inulase and perhaps some of the other enzymes would be produced because the substances which they transform are present in the mycelium. If the action of an enzyme is reversible and they are synthesizing agents, then the question arises: "can the products of the decomposition induce the formation of enzyme?" In my experiments the only substance besides tannic acid capable of inducing the formation of the tannase is gallic acid, which is a decomposition product of tannic acid.

Might it be possible that all of the enzymes are produced only in response to the influence of the zymolyte or to the products of its decomposition present either in the nutrient solution or in the mycelium? There is a considerable amount of evidence indicating that one or the other is always present, but there is also evidence that certain enzymes are seemingly produced in the entire absence of the zymolyte or the products of its decomposition. The whole problem is a complex one and requires investigation.

³⁵ Enzyme Studies of Lower Fungi, *loc. cit.*

METHOD OF PREPARING ASH-FREE CASEIN AND PARACASEIN.

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Casein or paracasein that is to be used in studying the relations of these compounds to mineral bases must be free from all such bases. The preparation of casein or paracasein really ash-free is much more difficult than has been commonly assumed. The so-called chemically pure casein furnished by chemical supply houses usually contains as much as 0.6 per cent of ash. The preparations used in various investigations in which the ash content has been reported rarely contain less than 0.2 per cent of ash and not infrequently more than 0.5 per cent.

The principal basic element in casein or paracasein preparations, as usually made, is calcium, and its presence is frequently due to the existence of a compound of calcium and casein containing 0.22 per cent Ca (equal to 0.31 per cent CaO) or, in case of paracasein, double this amount of Ca, as we shall show in another paper (this *Journal*, p. 223). These salts of casein and paracasein are insoluble in water but easily soluble in a 5 per cent solution of NaCl, while base-free casein and paracasein are insoluble both in water and in the brine solution.

When casein or paracasein is carefully precipitated by dilute acids from milk or from their lime-water solutions, the precipitate is apt to contain more or less of the above-mentioned caseinate or paracaseinate in addition to base-free protein. The precipitation of such calcium salts occurs most readily when the usual precautions in precipitating casein or paracasein from milk are most rigidly observed, that is, when excess of acid is avoided. We have examined casein preparations obtained from chemical supply houses and have found that some of them are soluble in 5 per cent solution of NaCl to the extent of 50 per cent or more of their weight.

PREPARATION OF ASH-FREE CASEIN.

After trying different methods of preparing casein so as to contain a minimum amount of calcium, we have obtained the most satisfactory results by the method described below. We have been able to prepare casein containing only 0.06 per cent of ash, consisting largely of calcium phosphate, derived from the trace of calcium not removed and the phosphorus of the casein molecule. The amount of calcium present in 5 grams of such material was too small to determine quantitatively.

Our method of preparation is to dilute separator skim-milk with seven or eight times its volume of distilled water and carefully add dilute acetic acid (6 cc. of glacial acetic acid diluted to 1 liter) until the casein separates completely; after which the clear solution is removed by siphon as soon as the precipitate settles. Distilled water is then added, the mixture stirred vigorously and the precipitate allowed to settle, after which the wash water is siphoned off. Water is again added and the casein is dissolved by adding, for each liter of milk used, 1 liter of dilute NH_4OH (6 cc. of strong reagent diluted to 1 liter). When the solution is complete, the whole is filtered through a thick layer of absorbent cotton. The casein is then precipitated again with dilute acetic acid; the precipitate is allowed to settle, and is then washed, redissolved in dilute NH_4OH and filtered, the process of precipitation, washing, dissolving, etc., being repeated not less than four times. Finally an excess of strong NH_4OH (10 cc.) is added and then, 20 cc. of saturated solution of ammonium oxalate. The mixture is allowed to stand twelve hours or more. Calcium is precipitated as oxalate in very finely divided condition, too fine to permit its satisfactory removal by ordinary methods of filtration. Better aggregation of the precipitate can, however, be effected by means of centrifugal force. The centrifuged mixture is then filtered through double thickness of filter paper. The filtered solution is next treated with dilute HCl (10 cc. HCl , sp. gr. 1.20, diluted to 1 liter) until the casein is precipitated. The precipitate is washed with distilled water until free from chloride and is then placed on a hardened filter paper in a Buchner funnel, as much water as possible being now removed by suction. The mass is next transferred to a large mortar and thoroughly triturated with 95 per cent alcohol. The

alcohol is then removed by suction on a Buchner funnel and the casein is then again placed in a mortar and triturated with absolute alcohol. Most of the alcohol is removed and the casein treated twice with ether in a mortar by trituration, the ether being removed each time by means of suction on a Buchner funnel. The material is then placed in a large evaporating dish and spread out in a layer as thin as possible; it is allowed to stand twelve hours or more in a warm place. The material is finally ground in a mortar until the particles pass a 40-mesh sieve and is dried for two days over H_2SO_4 in a desiccator under diminished pressure.

Three preparations made in this way were found to show an ash content of 0.10, 0.09 and 0.06 per cent, respectively. These preparations were insoluble in water and in 50 per cent alcohol; the first one was very slightly soluble in a 5 per cent solution of NaCl , but the two others were not.

When 1 gram of these casein preparations was treated with 10 cc. of $\frac{N}{10}$ NH_4OH , NaOH or KOH and 90 cc. of water, a clear solution was obtained, the casein dissolving completely. When to this solution a minute amount of a solution of a salt of barium, strontium or calcium was added, there developed promptly the opalescent appearance characteristic of casein solutions under such conditions.

Casein prepared in the manner described was analyzed with the following results: Moisture, 1.09 per cent. In the dry substance: Ash, 0.06; C, 53.50; H, 7.13; N, 15.80; P, 0.71; S, 0.72; O (by difference), 22.08 per cent.

PREPARATION OF ASH-FREE PARACASEIN.

Separator skim milk was heated to 37°C . and rennet extract (Hansen's) was added in the proportion of 0.12 cc. per 1000 cc. of milk. The mixture was allowed to stand until the precipitated paracaseinate had separated as completely as possible. The resulting curd was then stirred vigorously in order to break it into small pieces and hasten the separation of whey. When the curd had settled, the supernatant whey was removed by siphon. The paracaseinate was washed with distilled water several times and finally 5 liters of water added for each liter of milk originally used. Dilute NH_4OH (6 cc. of strong reagent diluted to 1000 cc.) was then added, as in the case of preparation of casein described above, and the mixture stirred until the paracaseinate was dissolved. The

process of reprecipitating, washing and redissolving was continued as in the case of casein; the remaining calcium was finally separated by addition of ammonium oxalate and centrifuging as described above.

One preparation made in this way contained 0.07 per cent. of ash. One gram gave a clear solution when dissolved in 10 cc. of $\frac{3}{4}$ NH_4OH and 90 cc. of water.

One preparation, with a high ash content, gave the following results when analyzed: Moisture, 1.63 per cent. In the dry substance, ash, 0.61; C, 53.50; H, 7.26; N, 15.80; P, 0.83; S, 0.87; O (by difference), 21.13.

Another preparation with exceptionally low ash content gave the following results: Ash, 0.07; phosphorus, 0.71; sulphur, 0.72 per cent.

SUMMARY.

Ash-free casein and paracasein are prepared by alternate precipitation with dilute acids and solution with dilute NH_4OH several times, the last portion of calcium being removed by precipitation with ammonium oxalate, after which the protein is precipitated with dilute acid and purified by treatment with water, alcohol and ether, being finally dried over H_2SO_4 under reduced pressure.

PREPARATION AND COMPOSITION OF BASIC CALCIUM CASEINATE AND PARACASEINATE.

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The compound commonly known as basic calcium caseinate contains the largest amount of calcium in combination with casein. This is the compound that has been most frequently prepared and studied by investigators; beginning with Söldner.¹ Varying results have been obtained by different workers, the percentage of Ca varying from 1.66 to 2.13 per cent (equal to 2.32 to 2.98 per cent CaO).

This compound can be prepared in two different ways: (1) By decomposing CaCO_3 with casein and (2) by treating casein with a solution of Ca(OH)_2 (lime-water).

Preparation of basic calcium caseinate by treating casein with CaCO_3 .

When casein is treated with CaCO_3 , the results of the reaction can be measured in two ways: (1) By weighing the CO_2 displaced and (2) by determining the amount of Ca in the resulting compound. Both methods were employed by us.

Ash-free casein prepared in the manner described in the previous article was placed in the flask of a Knorr CO_2 apparatus and an excess of CaCO_3 suspended in water was added. The CO_2 formed in the reaction was run into weighed bulbs containing KOH, and the increase of weight due to CO_2 determined at the end of the reaction. The results are given in Table I.

For the purpose of measuring the results of the reaction by determining the amount of Ca in the resulting compound, the casein was placed in a mortar and thoroughly triturated with an excess

¹ Landw. Versuchsstat., xxxv, p. 351, 1888.

TABLE I.

Amounts of CO₂ expelled from CaCO₃ by casein.

AMOUNT OF DRY CASEIN USED	AMOUNT OF CO ₂ EXPELLED	CaO (AND Ca) FOR 100 GRAMS OF CASEIN, EQUIVALENT TO CO ₂
grams	grams	grams
10	0.1900	2.42 (1.73 Ca)
10	0.1980	2.52 (1.80 Ca)
5	0.1054	2.68 (1.91 Ca)
5	0.1003	2.55 (1.81 Ca)
Average.....		2.54 (1.81 Ca)

of moist CaCO₃, the excess being removed by filtration at the end of the reaction. The filtrate was treated with 95 per cent alcohol, which was acid-free, until the calcium caseinate was precipitated, after which the precipitate was washed with alcohol and ether and dried at 120°C. A weighed portion of this compound was carefully ignited and the Ca in the resulting ash was determined, with the following results:

TABLE II.

Amount of Ca combining with casein when reacting with CaCO₃.

WEIGHT OF CASEINATE	WEIGHT OF CaO	WEIGHT OF Ca	WEIGHT OF FREE CASEIN	CaO (AND Ca) FOR 100 GRAMS OF CASEIN
grams	grams	grams	grams	grams
0.4125	0.0102	0.0073	0.4052	2.52 (1.80 Ca)
0.5134	0.0124	0.0089	0.5045	2.46 (1.76 Ca)
0.3090	0.0077	0.0055	0.3035	2.54 (1.81 Ca)
0.4253	0.0104	0.0074	0.4179	2.49 (1.77 Ca)
Average..0.41505	0.010175	0.00726	0.4078	2.50 (1.78 Ca)

Preparation of basic calcium caseinate by treating casein with an excess of Ca(OH)₂.

Weighed portions of casein were dissolved in an excess of Ca(OH)₂ solution. Phenolphthalein indicator was then added to the solution and HCl was run in until the solution became neutral. The solution was then dialyzed to remove the CaCl₂ formed in neutralization. The dialyzed solution was evaporated to dryness, the residue dried at 120°C. and weighed. The determination of Ca was made, after ignition, with the following results:

TABLE III.

Amount of Ca combining with casein on treatment with Ca(OH)₂.

WEIGHT OF CASEINATE	WEIGHT OF CaO	WEIGHT OF Ca	WEIGHT OF FREE CASEIN	CaO (AND Ca) FOR 100 GRAMS OF CASEIN
grams	grams	grams	grams	grams
1.582	0.040	0.0286	1.5534	2.58 (1.84 Ca)
1.471	0.035	0.0250	1.4460	2.42 (1.73 Ca)
1.548	0.038	0.0271	1.5209	2.50 (1.78 Ca)
Average..1.534	0.0377	0.0269	1.5070	2.50 (1.78 Ca)

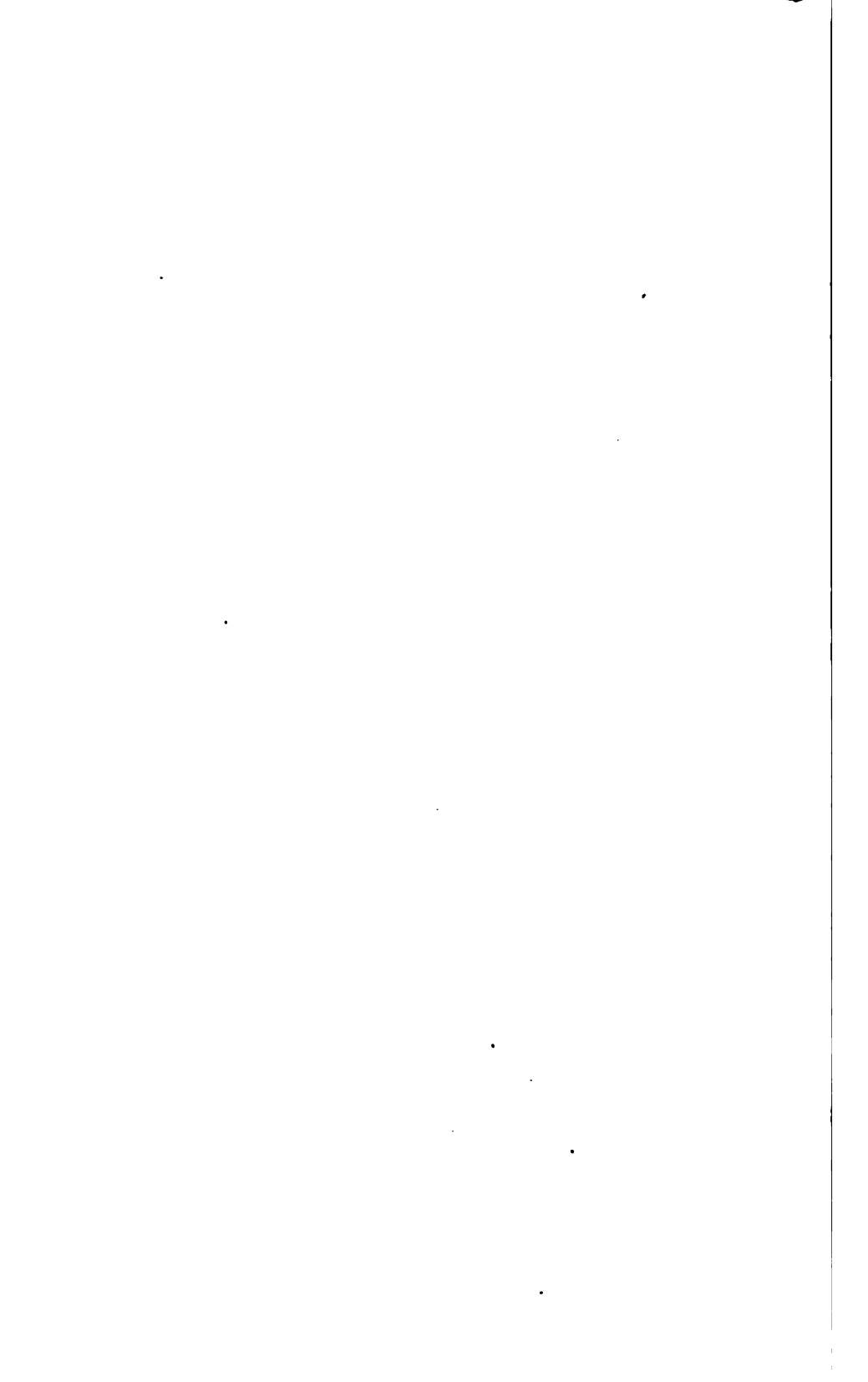
The three sets of figures presented in tables I, II and III indicate that casein combines with Ca to form a compound containing about 2.50 per cent of CaO (equal to 1.78 per cent of Ca); the compound in solution is neutral to phenolphthalein. Expressed in another form, 1 gram of casein combines with 9×10^{-4} gram equivalents of Ca.

Composition of basic calcium paracaseinate.

Like casein, paracasein manifests its acid character by its power to liberate CO₂ from CaCO₃, forming a calcium paracaseinate. The results of the reaction were measured by us, in the manner described above, in the case of casein. The average of many determinations indicates that paracasein unites with Ca to form a paracaseinate which is neutral to phenolphthalein and has the same general composition as the caseinate.

SUMMARY.

Basic calcium caseinate and paracaseinate are prepared (a) by treating the ash-free protein with CaCO₃ and (b) by dissolving protein in Ca(OH)₂ and neutralizing to phenolphthalein with HCl. In the first reaction, the amount of CO₂ displaced by the protein was determined and also the amount of Ca in the compound resulting. In the second reaction; the resulting compound was isolated, purified and its Ca content determined. The different sets of determinations agree in showing the compound to contain about 2.50 per cent of CaO (equal to 1.78 per cent of Ca) or 1 gram of protein combines with 9×10^{-4} gram equivalents of Ca.



PREPARATION AND COMPOSITION OF UNSATURATED OR ACID CASEINATES AND PARACASEINATES.

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Compounds of casein with bases, in which less base is present than in basic calcium caseinate (this *Journal*, p. 207), have been reported. Söldner¹ obtained a compound of casein and Ca containing 1.11 per cent of Ca (equal to 1.55 per cent of CaO); or, expressed in another form, 1 gram of casein combined with 5.55×10^{-4} gram equivalents of Ca. This compound is neutral to litmus but acid to phenolphthalein and has been commonly known as *neutral calcium caseinate*. This compound as prepared by Van Slyke and Hart² contains about 1.07 per cent of Ca (equal to about 1.50 per cent of CaO); or, 1 gram of casein combines with 5.35×10^{-4} gram equivalents of calcium. Courant³ believes that, in addition to the basic and neutral compounds of casein and Ca, a third exists, in which the Ca is present in about one-half the amount contained in the neutral compound and one-third that contained in the basic compound; he regards them as mono-, di-, and tri-calcium caseinates. Timpe⁴ reports a compound containing 0.961 per cent of Na (equal to 0.868 per cent of CaO or 0.62 per cent of Ca); or, 1 gram of casein combines with 3.1×10^{-4} gram equivalents of Ca. Long⁵ was able to dissolve 1 gram of casein in just one-half the amount of alkali required for the phenolphthalein neutralization and therefore inferred the existence of acid caseinates containing one-half the amount of base contained in

¹ *Landw. Versuchsstat.*, xxxv, p. 351, 1888.

² *Amer. Chem. Journ.*, xxxiii, p. 461, 1905.

³ *Pflüger's Archiv f. d. ges. Physiol.*, p. 109, 1891.

⁴ *Arch. f. Hyg.*, xviii, p. 1, 1893.

⁵ *Journ. of Amer. Chem. Soc.*, xxviii, p. 372, 1906.

basic calcium caseinate. The existence of such a combination is questioned by Robertson.⁶

In the course of our work, we became convinced that casein forms compounds containing less base than any of those reported by other workers. While we were at work on this point, an article by Robertson⁷ appeared in which was reported a combination of casein and NaOH, 1 cc. of alkali combining with 0.877 gram of casein. Our further work confirms Robertson's results, although we have used a different method of procedure. In addition, we have been able to prepare and isolate several salts for analysis. Our study of these individual salts shows that NH_4 , Na and K compounds possess properties very different from those of Ba, Ca and Sr. We have prepared and studied two sets of compounds of casein with bases, in one of which 1 gram of casein combines approximately with 1.125×10^{-4} gram equivalents of base, while in the other 1 gram of casein combines with about 2.25×10^{-4} gram equivalents of base.

We shall now take up the details of our experimental work in preparing unsaturated or acid caseinates of the bases of the more common alkalies and alkaline earths.

The specific object in view was to ascertain the smallest quantity of base with which casein combines to form a definite salt. In the volumetric work our method of procedure was as follows:

In 200 cc. of $\frac{N}{10}$ alkali, we dissolved 5 grams of pure casein as quickly as possible and then made the volume to 250 cc. Each 50 cc. of this solution therefore represented 1 gram of casein dissolved in 50 cc. of $\frac{N}{10}$ alkali. A preliminary or trial determination was next made in the following manner. Into a 300 cc. Erlenmeyer flask we measure 50 cc. of the caseinate solution and then add, a drop at a time, some $\frac{N}{10}$ HCl, until we have used 5 cc., the contents of the flask being kept in constant agitation in order to prevent premature precipitation of casein. After addition of 5 cc. of acid, a portion of the contents of the flask is centrifuged, in order to cause the sedimentation of precipitated casein, if any, a precipitate serving as an indicator. A sedimentation tube of 50 cc. capacity can be used; the precipitate collects in the lower V-shaped portion. It is possible in this manner to detect the casein precipitated by 0.20 cc. of $\frac{N}{10}$ HCl. In case no casein is precipitated by the first addition of 5 cc. of acid, another equal amount of acid is added and a portion of the mixture centrifuged; the process of adding

⁶ This *Journal*, ii, p. 336, 1906.

⁷ *Journ. of Physical Chem.*, xiii, p. 469, 1909.

5 cc. portions of acid and centrifuging is continued until a permanent precipitate of casein is obtained. This shows, within 5 cc. of $\frac{N}{50}$ HCl, how much acid is required to start definite precipitation of the casein. In order to ascertain the exact point more closely, another set of determinations is made, using 50 cc. of the caseinate solution and adding in the same continuous manner an amount of $\frac{N}{50}$ HCl which is 5 cc. less than the amount causing the first appearance of a permanent precipitate in the trial or preliminary determination. The acid is now added in small amounts with constant agitation of the liquid in order to prevent the premature separation of any precipitate, and centrifuged after the addition of each 0.25 cc. The first point at which a permanent precipitate appears is noted; the addition of acid is continued until all of the casein is precipitated and this point is also noted. In our work this method of determination was repeated several times with each combination of casein and alkali and three different casein preparations were used in preparing each caseinate.

We shall now present the results of our experimental work in connection with unsaturated or acid caseinates of, first, NH_4 , Na and K and, second, Ca, Sr and Ba.

Acid caseinates of NH_4 , Na and K.

In the manner described above, we made numerous determinations in the case of preparations of 1 gram of base-free casein dissolved in 50 cc. of the hydroxides of NH_4 , Na and K, respectively. In every case, irrespective of the alkali used, the volume of $\frac{N}{50}$ HCl required to cause the first sign of permanent precipitation was between 44.25 and 44.50 cc.: in each case also the amount necessary to cause complete precipitation was 50 cc.

These results indicate that 1 gram of casein forms a soluble compound with NH_4 , Na and K, when combined with amounts of each somewhere between 1.10×10^{-4} and 1.15×10^{-4} gram equivalents, expressed as hydroxide; or, expressed in another form, 1 cc. of $\frac{N}{10}$ alkali combines with an amount of casein somewhere between 0.87 and 0.91 gram. The proportion of basic element in each compound is approximately the following: NH_4 , 0.20 per cent; Na, 0.26 per cent; and K, 0.44 per cent. Caseinates combining with the amount of alkali base indicated contain the smallest known amount of base, according to our present knowledge. It seems proper, therefore, to suggest that such compounds be called *mono-basic caseinates*.

Preparation of mono-ammonium caseinate. It seemed desirable that we should carry the work somewhat further and prepare one

pure compound, at least, in dry form for study. The NH_4 compound was chosen as the one offering least difficulty. The method of preparation was as follows:

In 2 liters of distilled water containing 250 cc. of $\frac{N}{10}$ NH_4OH , 25 grams of base-free casein were dissolved. After solution was complete, we slowly added 125 cc. of $\frac{N}{10}$ HCl , care being taken to agitate the mixture during the addition of the acid, in order to prevent premature precipitation of any casein. There was next added very cautiously $\frac{N}{10}$ HCl until a permanent precipitate began to appear, as shown by centrifuging the mixture at intervals. The solution was then filtered and measured. The amount of $\frac{N}{10}$ HCl required to precipitate the casein completely was determined in an aliquot part. Then one-third of this amount was added to insure the presence of only mono-basic caseinate. Any precipitate formed was removed by filtration and the filtrate was dialyzed until the NH_4Cl that had been formed in the reaction was completely removed. The resulting solution, containing mono-ammonium caseinate, was then precipitated by addition of acid-free alcohol. The precipitate was filtered, washed with acid-free alcohol and ether and dried at 120°C . In several preparations thus made, the amount of NH_4 was determined; the results are given in the following table:

TABLE I.
Composition of mono-ammonium caseinate.

AMOUNT OF CASEINATE USED	AMOUNT OF $\frac{N}{10}$ NH_4OH FOUND	RELATION OF CASEIN TO NH_4OH IN CASEINATE	PERCENTAGE OF NH_4 IN CASEINATE
grams	cc.		
5.891	6.64	1 gm. casein to 1.127×10^{-4} gm. equivalents	0.203
4.870	5.38	1 gm. casein to 1.105×10^{-4} gm. equivalents	0.200
*4.000	4.30	1 gm. casein to 1.075×10^{-4} gm. equivalents	0.194
*3.000	3.16	1 gm. casein to 1.053×10^{-4} gm. equivalents	0.190

* Preparations of caseinates made by Mr. O. B. Winter.

Acid caseinates of Ca, Sr and Ba.

In making preparations of the caseinates of the alkali earth bases, difficulty was experienced for some time in obtaining concordant results. The trouble was finally found to be due to the presence of the chloride formed when the solution of the caseinate is treated with HCl . Such chlorides tend to cause precipitation

of the caseinates either by decreasing their solubility or, perhaps, by formation of double salts, consisting of the chloride in combination with the caseinate.⁸ The difficulty of insolubility is readily overcome by removal of the chloride through simple dialysis before its amount is sufficient to cause precipitation. To accomplish this we made use of the following process.

In 200 cc. of $\frac{N}{10}$ hydroxide of Ca, Sr or Ba, we dissolved 5 grams of casein and then diluted the solution to 250 cc. A trial or preliminary determination was made by adding $\frac{N}{50}$ HCl to 50 cc. of the caseinate solution in portions of 5 cc. at a time, agitating constantly and, after each addition, testing for the presence of a precipitate by centrifuging a portion, until a precipitate appeared, just as in the case of preparing alkaline caseinates (p. 212). Then to each of several flasks containing 50 cc. of the caseinate solution, we added an amount of $\frac{N}{50}$ HCl that was 5 cc. less than the amount causing the first appearance of a permanent precipitate in the preliminary trial. The contents of the flask were then placed in dialyzing tubes, and, by frequent changes of the surrounding water, most of the soluble chloride that had been formed was removed. The contents of one tube were used for another preliminary test. An amount of acid less than that required to produce a precipitate in this second test was then added to all the tubes and the contents again dialyzed. This operation was continued in the manner indicated in Table 216 (p. 216):

In the manner described above, we have made numerous preparations of Ca, Sr and Ba caseinates; the averages of many results show that, in adding $\frac{N}{50}$ HCl to 50 cc. of a caseinate solution containing 1 gram of casein dissolved in 50 cc. of $\frac{N}{50}$ solution of hydroxide of Ca, Sr and Ba, 38.5 to 39.0 cc. of $\frac{N}{50}$ HCl will be required to cause the first appearance of a permanent precipitate; also, the addition of only 44.5 cc. will be required to cause the complete precipitation of the casein. The remaining amount of base, equal to 5.5 cc. of $\frac{N}{50}$ hydroxide, or 1.1 cc. of $\frac{N}{10}$ hydroxide, appears to be held in combination in the insoluble compound.

These results indicate the formation of two sets of compounds, when casein is dissolved in a hydroxide of Ca, Sr or Ba and this solution is neutralized with acid under the conditions of our experiments. One set of compounds contains twice as much basic element as the other.

Attention is called to additional details in the following statements:

⁸ Pfeiffer and Modelski: *Zeitschr. f. physiol. Chem.*, lxxx, p. 329, 1912.

1. In the di-basic compounds, as the results show, 1 gram of casein requires between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents of hydroxide of Ca, Sr or Ba to form a compound which is soluble in water when there is not present any, or more than a trace of, soluble chloride of any of these elements. The addition

TABLE II.

Illustration of method used in preparing acid caseinates of Ca, Sr and Ba.

AMOUNT OF CASEIN IN SOLUTION	AMOUNT OF N 50 HYDROXIDE SOLUTION USED	AMOUNT OF N 50 HCL ADDED	SIGN OF FIRST PERMANENT PRECIPITATE	
gram	cc.	cc.		
1	50	30	precipitate	First trial
1	50	25	0	Dialyzed and used for next.
1	50	30	0	Dialyzed and used for next.
1	50	35	precipitate	
1	50	25	0	Dialyzed and used for next.
1	50	30	0	Dialyzed and used for next.
1	50	35	0	
1	50	40	precipitate	
1	50	25	0	Dialyzed and used for next.
1	50	30	0	Dialyzed and used for next.
1	50	35	0	Dialyzed and used for next.
1	50	36	0	
1	50	37	0	
1	50	38	precipitate	
1	50	25	0	Dialyzed and used for next.
1	50	30	0	Dialyzed and used for next.
1	50	35	0	Dialyzed and used for next.
1	50	37	0	Dialyzed and used for next.
1	50	38	0	
1	50	39	precipitate	
1	50	25	0	Dialyzed and used for next.
1	50	30	0	Dialyzed and used for next.
1	50	35	0	Dialyzed and used for next.
1	50	37	0	Dialyzed and used for next.
1	50	38	0	Dialyzed and used for next.
1	50	38.5	0	
1	50	39	precipitate	

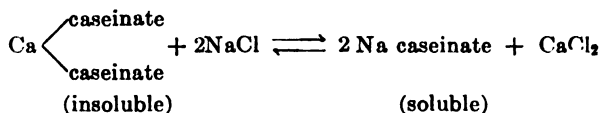
of even a small amount of a soluble salt of any of these elements to a solution of any of these di-basic caseinates causes the formation of a precipitate.

2. In these di-basic compounds, 100 grams of casein combine (a) with 0.44 to 0.46 gram of Ca (equal to 0.62 to 0.64 gram of CaO), (b) with 0.96 to 1.01 grams of Sr (equal to 1.14 to 1.19 grams of SrO) or (c) with 1.51 to 1.58 grams of Ba (equal to 1.69 to 1.76 grams of BaO).

3. It is indicated that with the treatment described above 1 gram of casein combines with about 1.1×10^{-4} gram equivalents of the hydroxide of Ca, Sr or Ba to form an *insoluble* compound, when an acid is added in amount just sufficient to precipitate the casein completely. These compounds are regarded as *mono-basic*.

4. In these insoluble mono-basic compounds, 100 grams of casein combine approximately (a) with 0.22 gram of Ca (equal to 0.31 gram of CaO), (b) with 0.48 gram of Sr (equal to 0.57 gram of SrO) or (c) with 0.76 gram of Ba (equal to 0.85 gram of BaO).

5. These insoluble compounds possess some highly interesting properties. They are soluble in a 5 per cent solution of NH_4Cl , NaCl and KCl . This solubility is due to an exchange of bases, which, for the purpose, can be represented by the following reversible reaction:



That the reaction is a reversible one is supported by the following experimental evidence: Mono-calcium caseinate was prepared and freed from soluble calcium salts by washing and dialysis. The compound was then dissolved in a 5 per cent solution of calcium-free NaCl . That an interchange of bases had taken place was shown by the fact, that when the brine solution of caseinate was dialyzed, calcium was found in the solution outside the dialyzing tube. This brine solution of caseinate was then dialyzed until free from calcium and was then filtered. A solution of CaCl_2 was then added to this dialyzed solution and at once a precipitate of calcium caseinate was produced. That this precipitate is a cal-

cium salt can be shown in two ways: (1) By washing and dialyzing until free from soluble chloride and then igniting; calcium is found in the ash. (2) By washing and dialyzing until free from soluble calcium, then redissolving in a 5 per cent solution of calcium-free NaCl and dialyzing; calcium is found to dialyze out of this brine solution of caseinate.

Preparation of mono- and di-calcium caseinates. In order to study the composition and properties of these compounds more fully, preparations of mono- and di-calcium caseinate were made. The following method was employed:

In 800 cc. of $\frac{N}{10}$ Ca(OH)₂ there were dissolved 20 grams of base-free casein. To this solution were added 400 cc. of $\frac{N}{10}$ HCl; the solution was then dialyzed to remove most of the resulting CaCl₂. Then $\frac{N}{10}$ HCl was added very cautiously under constant agitation of the mixture until a permanent precipitate began to appear, as shown by centrifuging. The solution was then dialyzed again and then more acid was added until a precipitate once more began to form. Alternate dialysis and addition of acid were continued until no more acid could be added without causing a precipitate. The amount of acid necessary to precipitate all of the casein was next determined in an aliquot portion, and one-third of this amount of acid was then added. The precipitated casein was filtered out and the filtrate was dialyzed. This solution contained di-calcium caseinate. The solution was divided, one portion being used for the preparation of di-calcium caseinate and the other for the mono-calcium caseinate.

In completing the preparation of the di-calcium caseinate, the salt was precipitated by addition of acid-free alcohol, the precipitate being washed in acid-free alcohol and ether, and then dried at 120°C. The composition of this preparation is given below in table IV.

In preparing the mono-calcium caseinate, the solution of di-calcium caseinate was treated with enough acid to precipitate three-fourths of the casein. The resulting precipitate was filtered, washed with water, acid-free alcohol and ether, and then dried at 120°C. The results in table III show the amount of Ca found in the preparation.

If we compare the results given in tables III and IV with the figures given in paragraphs (1), (2), (3) and (4) on page 217 it is obvious that the results embodied in these tables are lower. The higher results are obtained by the volumetric method and are believed to be nearer the truth, owing to the difficulty of preparing these caseinates in pure form. The values given by the volumetric method are the following: 1 gram of casein to 1.10 (to 1.15) $\times 10^{-4}$ gram equivalents of calcium for the mono-basic

TABLE III.

Composition of mono-calcium caseinate preparation.

AMOUNT OF COM- POUND USED	AMOUNT OF CaO (AND Ca) FOUND	PERCENTAGE OF CaO (AND Ca) IN COMPOUND	RELATION OF CASEIN TO CALCIUM IN COMPOUND
grams	gram		
5	0.0149 (0.0106Ca)	0.298 (0.213 Ca)	1 gm. casein to 1.06×10^{-4} gm. equivalents.
5	0.0141 (0.0101Ca)	0.282 (0.201 Ca)	1 gm. casein to 1.01×10^{-4} gm. equivalents.
5	0.0146 (0.0104Ca)	0.292 (0.209 Ca)	1 gm. casein to 1.04×10^{-4} gm. equivalents.
Average...	0.01453 (0.0104Ca)	0.291 (0.208 Ca)	1 gm. casein to 1.04×10^{-4} gm. equivalents.

TABLE IV.

Composition of di-calcium caseinate preparation.

AMOUNT OF COM- POUND USED	AMOUNT OF CaO (AND Ca) FOUND	PERCENTAGE CaO (AND Ca) IN COMPOUND	RELATION OF CASEIN TO CALCIUM IN COMPOUND
grams	gram		
4.2825	0.0233 (0.0167Ca)	0.544 (0.39 Ca)	1 gm. casein to 1.95×10^{-4} gm. equivalents.
4.1215	0.0235 (0.0168Ca)	0.572 (0.41 Ca)	1 gm. of casein to 2.04×10^{-4} gm. equivalents.
Average 4.202	0.0234 (0.01675Ca)	0.558 (0.40 Ca)	1 gm. of casein to 2.00×10^{-4} gm. equivalents.

caseinate, and 1 gram of casein to 2.2 (to 2.3) $\times 10^{-4}$ gram equivalents of calcium for the di-basic caseinate.

PREPARATION AND COMPOSITION OF UNSATURATED OR ACID PARACASEINATES.

In preparing acid paracaseinates of bases, the same volumetric method of procedure was followed as in case of the casein salts (p. 215). The appearance of a precipitate in a centrifuged portion after addition of acid to an alkali solution of paracaseinate was made to serve as an indicator in regard to the end point of the reaction. We dissolved 5 grams of the ash-free paracaseinate in 200 cc. of $\frac{N}{10}$ alkali, made up the solution to 250 cc. and then brought to the end point by careful addition of $\frac{N}{10}$ HCl.

Acid paracaseinate of NH₄, Na and K.

In the manner described, determinations were made in the case of base-free paracasein dissolved in hydroxides of NH₄, Na and K. In every case, irrespective of the alkali used, the volume of $\frac{N}{10}$ HCl required to cause the first sign of permanent precipitation when added to a solution of 1 gram of casein in 50 cc. of $\frac{N}{10}$ alkali was between 38.5 and 39 cc.; in each case, also, the amount required to cause complete precipitation was 50 cc.

These results show that 1 gram of paracasein combines with an amount of alkali somewhere between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents, expressed as hydroxide, in forming soluble compounds with NH₄, Na and K, which are acid to both litmus and phenolphthalein. Expressed in another form, 1 cc. of $\frac{N}{10}$ alkali, expressed as hydroxide, combines with an amount of paracasein somewhere between 0.435 and 0.455 gram. The proportion of basic element in each compound is approximately as follows: NH₄, 0.40 per cent; Na, 0.52 per cent; K, 0.88 per cent. The amount of each basic element in these paracaseinates is just double that present in the corresponding casein compounds.

The amount of acid required to precipitate completely the paracasein in these compounds is exactly equal to the alkali used to dissolve the paracasein; this fact indicates that there is not an additional paracaseinate, in insoluble form, containing less of these basic elements.

Preparation of mono-ammonium paracaseinate. Mono-ammonium paracaseinate was isolated and prepared in dry form for further study in the manner already described in the preparation of mono-ammonium caseinate (p. 213). Care must be taken to use a paracasein preparation free from casein or salts of Ca, Sr, Ba, etc. A determination of the amount of NH₄ present in preparations thus made is given in Table V.

These results illustrate the difficulty of preparing the compounds pure, but indicate that the percentage of NH₄ is about double that found in the corresponding mono-ammonium caseinate.

Acid paracaseinates of Ca, Sr and Ba.

In preparing paracasein salts of Ca, Sr and Ba, the presence of their chlorides causes much more trouble in respect to precipitation

TABLE V.

Composition of mono-ammonium paracaseinate.

AMOUNT OF PARACASEIN USED	AMOUNT OF $\frac{N}{10}$ NH ₄ OH FOUND	RELATION OF PARACASEIN TO NH ₄ OH IN PARACASEINATE	PERCENT- AGE OF NH ₄ IN PARACASE- INATE
4	8.20	1 gm. paracasein to 2.05×10^{-4} gm. equivalents.	0.37
4	7.98	1 gm. of paracasein to 2.00×10^{-4} gm. equivalents.	0.36

than in case of the caseinates. Special care must be taken to prevent the accumulation of chlorides of these elements. By sufficiently frequent dialysis, it was possible to obtain the results reported below. Another point in connection with paracasein is the fact of its slow rate of solution in the hydroxides of Ca, Sr and Ba; on this account we used 400 cc. of $\frac{N}{10}$ hydroxide to dissolve 5 grams of paracasein, making the volume up to 500 cc. with water.

Trial or preliminary determinations were made in the same manner as with casein (p. 215), in order to determine the amount of $\frac{N}{10}$ HCl required to precipitate the paracasein in the absence of CaCl₂, SrCl₂ or BaCl₂. The specific details employed and results obtained are indicated in Table VI (p. 222).

The results obtained by the method of procedure indicated above showed that the amount of $\frac{N}{10}$ HCl which must be added to a solution of 1 gram of paracasein in 100 cc. of $\frac{N}{10}$ hydroxide of Ca, Sr or Ba in order to cause the first sign of permanent precipitation was between 77.25 and 77.5 cc.: to completely precipitate all of the paracasein, 88.5 cc. were required. The figures are the same irrespective of the hydroxide used.

These results indicate the formation of two sets of compounds when paracasein is dissolved in a Ca(OH)₂, Sr(OH)₂ or Ba(OH)₂ and this solution is neutralized with acid under the conditions of our experiments. One set of compounds contains twice as much base as the other, corresponding to the two sets of casein compounds. The following statements call attention to additional details.

1. In the di-basic compounds, the results show that 1 gram of paracasein requires between 4.5×10^{-4} and 4.55×10^{-4} gram

TABLE VI.

Illustration of method of preparing acid paracaseinates of Ca, Sr and Ba.

AMOUNT OF PARACASEIN IN SOLUTION	AMOUNT OF N 55 HYDROXIDE SOLUTION USED	AMOUNT OF N 55 HCL ADDED	PRECIPITATION	
gram	cc.	cc.		
1	100	80.0	precipitate	First trial.
1	100	75.0	0	Dialyzed and used for next.
1	100	80.0	precipitate	
1	100	75.0	0	Dialyzed and used for next.
1	100	76.0	0	Dialyzed and used for next.
1	100	77.0	precipitate	
1	100	75.0	0	Dialyzed and used for next.
1	100	76.0	0	Dialyzed and used for next.
1	100	76.5	0	Dialyzed and used for next.
1	100	77.0	precipitate	
1	100	75.0	0	Dialyzed and used for next.
1	100	76.0	0	Dialyzed and used for next.
1	100	76.5	0	Dialyzed and used for next.
1	100	77.0	0	Dialyzed and used for next.
1	100	77.5	precipitate	
1	100	75.0	0	Dialyzed and used for next.
1	100	76.0	0	Dialyzed and used for next.
1	100	76.5	0	Dialyzed and used for next.
1	100	77.0	0	Dialyzed and used for next.
1	100	77.25	0	Dialyzed and used for next.
1	100	77.50	precipitate	

equivalents of hydroxide of Ca, Sr or Ba to form a compound which is soluble in pure water. These compounds are easily precipitated from their water solutions by a minute amount of a soluble salt of Ca, Sr or Ba.

2. In these di-basic compounds, 100 grams of paracasein combine approximately (a) with 0.90 gram of Ca (equal to 1.26 grams of CaO), (b) with 1.97 grams of Sr (equal to 2.33 grams of SrO) or (c) with 3.09 grams of Ba (equal to 3.45 grams of BaO).

3. It is indicated that, with the treatment described above,

1 gram of paracasein combines with about 2.3×10^{-4} gram equivalents of the hydroxide of Ca, Sr or Ba to form an *insoluble* compound. These compounds are regarded as mono-basic paracaseinates.

4. In these insoluble mono-basic paracaseinates, 100 grams of paracasein combine approximately (a) with 0.46 gram of Ca (equal to 0.64 gram of CaO), (b) with 1.01 grams of Sr (equal to 1.19 grams of SrO) or (c) with 1.58 grams of Ba (equal to 1.76 grams of BaO).

5. Mono-basic paracaseinates of Ca, Sr and Ba are completely soluble in warm 5 per cent solution of NH_4Cl , NaCl and KCl. This solubility is due to interchange of bases, just as in the case of caseinates (p. 217); the reaction was studied experimentally with paracaseinates and the same results obtained as in the case of the caseinates.

6. A comparison of the composition of the caseinates and paracaseinates shows that twice as much base is present in paracaseinates as in the corresponding caseinates. This is easily seen in the following table:

TABLE VII.

Comparison of composition of caseinates and paracaseinates.

Amount of basic element combined with 100 grams of casein or paracasein.

BASIC ELEMENT	IN MONO-BASIC CASEINATE	IN MONO-BASIC PARACASEINATE	IN DI-BASIC CASEINATE	IN DI-BASIC PARACASEINATE
Ca.....	0.22	0.46	0.44 to 0.46	0.90
Sr.....	0.48	1.01	0.96 to 1.01	1.97
Ba.....	0.76	1.58	1.51 to 1.58	3.09

Preparation of mono- and di-calcium paracaseinates. In order to study the composition and properties of these compounds further, preparations of the mono- and di-calcium paracaseinates were made. The first steps in making these compounds are the same. An excess of ash-free paracasein is agitated with lime-water until a saturated solution is formed, the undissolved paracasein being removed by filtration. To the solution, $\frac{N}{10}$ HCl is added until a permanent precipitate begins to appear. The solution is again filtered and then dialyzed. Alternate addition of acid and dialysis are continued until no more acid can be added after dialysis without causing precipitation. The amount of $\frac{N}{10}$ HCl required to

precipitate all the paracasein is next determined in an aliquot portion, and one-third that amount of acid is added. The solution is then filtered and dialyzed. This solution contains di-calcium paracaseinate. This solution is divided into two portions: in one the di-calcium paracaseinate is precipitated by addition of acid-free alcohol, the precipitate being washed with acid-free alcohol and ether and being dried at 120°C. This preparation was found to contain between 4.2×10^{-4} and 4.6×10^{-4} gram equivalents of Ca for 1 gram of paracasein.

In the second portion of di-calcium paracaseinate solution enough $\frac{N}{10}$ HCl is very slowly added to precipitate three-fourths of the paracasein in solution. The precipitate is mono-calcium paracaseinate; this is filtered, washed with acid-free alcohol and ether and dried at 120°C. Before being washed with alcohol, the precipitate is completely soluble in 5 per cent solution of NaCl. This compound, mono-calcium paracaseinate, is identical in its properties with the brine-soluble compound formed in Cheddar cheese, to which attention was first called by Van Slyke and Hart under the expression, "salt-soluble compound." Attention will be more fully called to this compound in another paper (this *Journal*, p. 231). An analysis of this preparation showed it to contain between 2×10^{-4} and 2.3×10^{-4} gram equivalents of Ca for 1 gram of paracasein.

SUMMARY.

1. Acid or unsaturated caseinates and paracaseinates of NH_4 , Na and K are prepared by dissolving the ash-free protein in $\frac{N}{10}$ hydroxide and neutralizing very carefully by successive additions with $\frac{N}{10}$ HCl, the end point being obtained by centrifuging a portion of the mixture in order to cause sedimentation of any precipitated protein; the desired end point is the first sign of a permanent precipitate. Protein precipitated by 0.20 cc. of $\frac{N}{10}$ HCl can thus be detected.

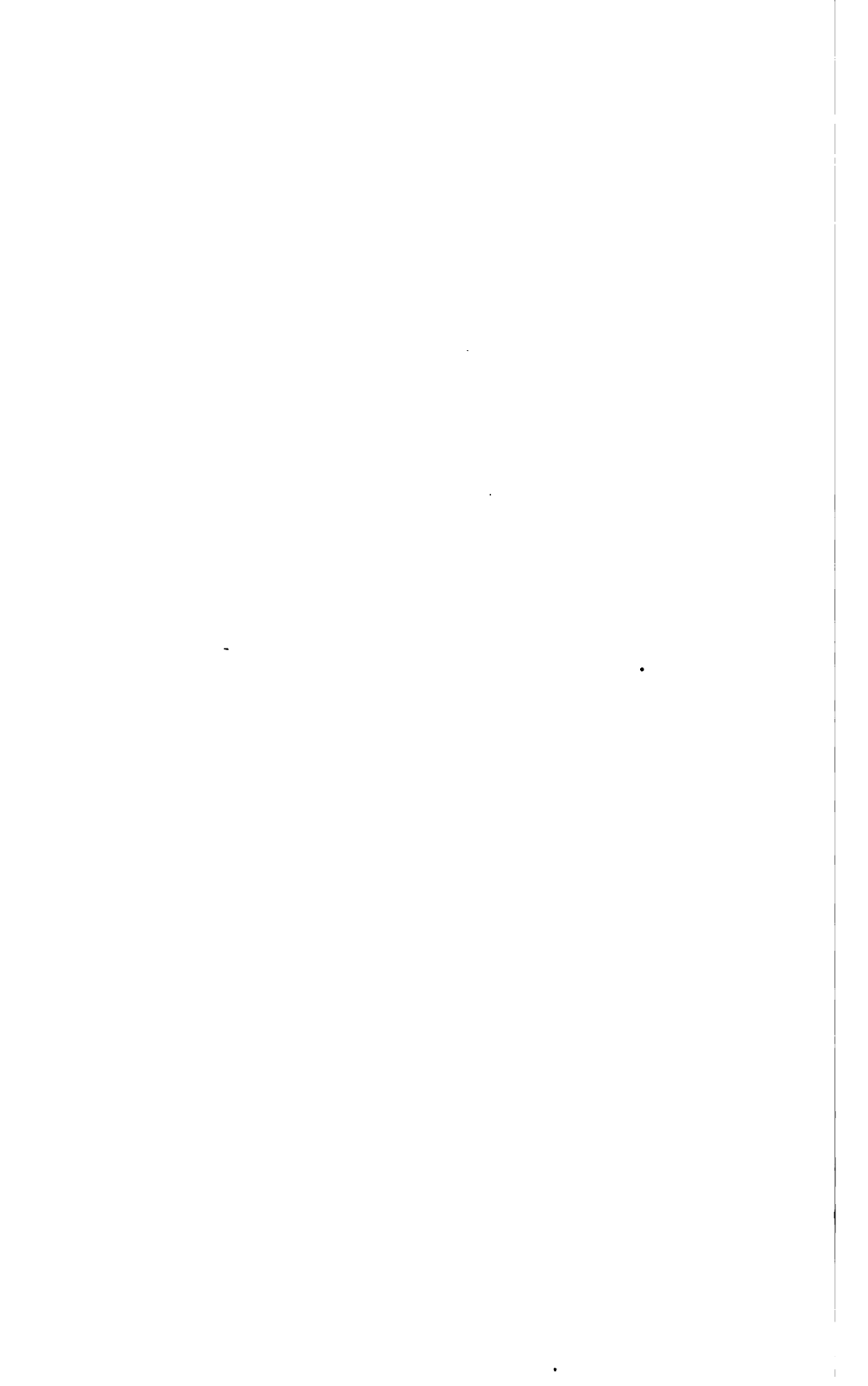
2. Results show that 1 gram of casein forms a soluble compound with NH_4 , Na or K, corresponding to amounts of hydroxide between 1.10×10^{-4} and 1.15×10^{-4} gram equivalents; or 1 cc. of $\frac{N}{10}$ alkali, expressed as hydroxide, combines with 0.87 to 0.91 gram of casein. Corresponding paracaseinates are formed but they contain twice the amount of basic element present in the caseinates.

Such compounds are called mono-basic. Mono-ammonium caseinates were prepared in dry form and studied.

3. Acid or unsaturated caseinates and paracaseinates of Ca, Sr and Ba are prepared by dissolving ash-free casein in $\frac{N}{10}$ hydroxide and proceeding as in case of alkali compounds, except that when the first sign of a permanent precipitate appears on the addition of HCl, the mixture is dialyzed to remove the chloride formed, because such chloride precipitates the caseinate or paracaseinate.

4. Two sets of compounds are formed with Ca, Sr and Ba, mono- and di-basic. In the di-basic caseinates, which are soluble, 1 gram of casein combines with 2.25 gram equivalents expressed as hydroxide. These compounds are easily precipitated by soluble salts of Ca, Sr or Ba. In the mono-basic caseinates, which are insoluble, 1 gram of casein combines with 1.125×10^{-4} gram equivalents expressed as hydroxide. In the paracaseinates, twice the amount of base combines with the protein molecule; 1 gram of paracasein combines with 4.50 gram equivalents expressed as hydroxide in the di-basic compounds and with 2.35 in the mono-basic.

5. Mono-basic caseinates and paracaseinates are insoluble in water but soluble in warm 5 per cent solution of NaCl, NH_4Cl , KCl, etc. The solubility is due to an exchange of bases; the reaction, for example, between mono-calcium caseinate and NaCl results in the formation of the soluble sodium caseinate and CaCl_2 . The reaction is reversible.



VALENCY OF MOLECULES AND MOLECULAR WEIGHTS OF CASEIN AND PARACASEIN.

By LUCIUS L. VAN SLYKE AND ALFRED W. BOSWORTH.

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(Received for publication, February 4, 1913.)

In the case of the compound of casein and Ca which is neutral to phenolphthalein, it is found that 1 gram of casein combines with 9×10^{-4} gram equivalents of Ca. In the case of the monoammonium caseinate, the combination is in the proportion of 1 gram of casein to a value between 1.1×10^{-4} and 1.15×10^{-4} gram equivalents expressed as hydroxide (this *Journal*, page 213.) Now, since we have one compound of known composition and another of approximately known composition, it should be possible by applying the rule of constant proportions to determine the true composition of the mono-basic caseinate and also the number of valencies satisfied in the caseinate that is neutral to phenolphthalein.

We have reason to believe that the proportion, 1 gram of casein to 1.125×10^{-4} gram equivalents of alkali hydroxide, is the true value, since, first, this lies between the two limits (1.10 and 1.15) found in our volumetric work; and, second, this figure agrees with that found by assuming a valency of 8 for the basic calcium caseinate, in which 1 gram of casein combines with 9×10^{-4} gram equivalents of Ca. Thus, if the valencies satisfied are 8, the proportion becomes 1 gram of casein to 1.125×10^{-4} gram equivalents of alkali, expressed as hydroxide, for monobasic caseinates. If, however, we were to assume that the number of valencies in the basic compound is 7 rather than 8, then the mono-basic salt would, theoretically, have the composition, 1 gram of casein to 1.285×10^{-4} gram equivalents of alkali, expressed as hydroxide, a value too high for our analytical results. If, on the other hand, we were to assume the numbers of valencies in the basic compound to be 9

(instead of 8), then the proportion in the mono-basic compound would become 1 gram of casein to 1×10^{-4} gram equivalents of alkali, expressed as hydroxide, a value too low for our analytical results obtained with mono-ammonium and other alkali caseinates. Therefore, assuming 8 as the true valency of basic calcium caseinate gives the value, 1 gram of casein to 1.125×10^{-4} gram equivalents of alkali, expressed as hydroxide, a result which agrees with the volumetric results obtained in the case of the mono-alkali caseinates.

Using the sulphur content as a basis on which to calculate the molecular weight of casein, we have $n \left(\frac{32.07}{0.72} \right) 100 = n4454+$.

If the value of n is 2, the molecular weight becomes 8908, which is in close agreement with the value previously found, 8888.

Using the amount of phosphorus in casein as a basis for calculating the molecular weight, we have $n \left(\frac{31.04}{0.71} \right) 100 = n4372-$, which becomes 8744 if the value of n is 2.

On the basis of 8 representing the true number of valencies satisfied in the basic calcium caseinate molecule, the molecular weight of casein is $\left(\frac{1}{1.125 \times 10^{-4}} \right)$ or 8888 +. Robertson reaches similar results¹ by deducing the molecular weight of casein in several different ways. This would also make the equivalent weight of casein equal to $\frac{8888}{8}$ or 1111. This value is in close agreement with the equivalent weight assigned by other workers to casein prepared from cow's milk. Laqueur and Sackur give about 1135,² Matthiopoulos gives 1131.5,³ Long gives 1124.⁴

As a result of the work done by us it would seem possible, theoretically, to prepare a series of not less than eight combinations of casein with each of the basic elements studied. According to what we have reason to believe at the present time, not less than four of these combinations have been prepared. Using the calcium compounds for illustration we have the following series:

¹ *Journ. of Physical Chem.*, xv, p. 179, 1911.

² *Hofmeister's Beiträge*, iii, p. 193, 1902.

³ *Zeitschr. f. anal. Chem.*, xlvii, p. 492, 1908.

⁴ *Journ. Amer. Chem. Soc.*, xxviii, p. 372, 1906.

NAME OF COMPOUND	GRAMS OF Ca FOR 100 GRAMS OF CASEIN	VALENCIES SATISFIED
Mono-calcium caseinate.....	0.22 (equal to 0.31 CaO)	1
Di-calcium caseinate.....	0.44 (equal to 0.62 CaO)	2
Neutral calcium caseinate.....	1.07 (equal to 1.50 CaO)	5
Basic calcium caseinate.....	1.78 (equal to 2.50 CaO)	8

It is noticeable that, in this series, compounds are absent representing valencies of 3, 4, 6 and 7. Whether such compounds can be prepared we cannot say at present.

VALENCY OF PARACASEIN MOLECULE AND MOLECULAR WEIGHT OF PARACASEIN.

In the case of basic calcium paracaseinate, the compound that is neutral to phenolphthalein, it is found that 1 gram combines with 9×10^{-4} gram equivalents of Ca, while in the case of mono-ammonium paracaseinate, the combination is in the ratio of 1 gram of paracasein to a value between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents (this *Journal* p. 223). According to the rule of constant proportions, the number of valencies satisfied in the first compound would be between $\frac{9}{2.2}$ and $\frac{9}{2.3}$ or 4. The molecular weight of paracasein would, therefore, be $\left(\frac{1}{2.25 \times 10^{-4}}\right)$ or 4444 +. Our results indicate that the molecular weight of casein, 8888, is just twice that of paracasein, 4444.

Calculated on the basis of the sulphur content, the molecular weight of paracasein would be $n \left(\frac{32.07}{0.72}\right) 100 = n4454+$; on the basis of the phosphorus content, we should have $n \left(\frac{31.04}{0.71}\right) 100 = n4372-$. The value of n would seem to be 1 and each molecule of paracasein would contain one atom each of sulphur and phosphorus.

Theoretically, it should be possible to make a series of four salts of paracasein. We have prepared three—those in which 1, 2 and 4 valencies are satisfied (pp. 219, 223).

In connection with the relative molecular weights of casein and paracasein, the facts indicate that the action of the principal

enzyme contained in rennet-extract splits the casein molecule into two molecules of paracasein, an effect opposite that advocated by some who believe that the paracasein molecule is a larger aggregation than that of casein. The following experiment supports our view:

Five grams of casein are dissolved in 250 cc. of $\frac{N}{50}$ -KOH. Using the volumetric method given elsewhere (this *Journal*, p. 212), it is found that 44.5 cc. of $\frac{N}{50}$ HCl could be added to 50 cc. of the caseinate solution, containing 1 gram of casein, before a permanent precipitate begins to appear. To another 50 cc. of caseinate solution a few drops of neutral rennet-extract are added. Under the conditions of the experiment, no precipitate or curd is produced by the action of the rennet-enzyme. After a few minutes, some $\frac{N}{50}$ HCl is added and it is found that a permanent precipitate begins to form as soon as we add only 39 cc. of $\frac{N}{50}$ HCl.

We have in hand a more extended investigation relating to the action of rennet-enzyme upon casein, the results of which will be published later.

SUMMARY.

On the basis of the analytical results obtained in the study of the composition of the mono-basic and basic caseinates and paracaseinates, the molecular weight of casein is 8388, that of paracasein, 4444. The valency of the protein molecule in basic caseinates is 8, in basic paracaseinates, 4.

COMPOSITION AND PROPERTIES OF THE BRINE-SOLUBLE COMPOUND IN CHEESE.

By LUCIUS L. VAN SLYKE AND ALFRED W. BOSWORTH.

(From the Chemical Laboratory of the New York State Agricultural Experiment Station, Geneva, N. Y.)

(Received for publication, February 4, 1913.)

During the manufacture and ripening of Cheddar cheese and of many other kinds of cheese, there is always found a protein that is soluble in a warm 5 per cent solution of NaCl. The existence of such a substance in Cheddar cheese was first brought to attention by work done in this laboratory.¹ The presence of this brine-soluble protein was shown to be associated in some way with the formation of acid in the cheese and, on the basis of some early experiments, VanSlyke and Hart were led to conclude erroneously that the substance consists of a combination of paracasein and lactic acid (called by them paracasein mono-lactate), which by the addition of more lactic acid becomes insoluble in dilute brine solution, forming a compound which they mistakenly regarded as paracasein di-lactate. As a result of later work,² they changed their first views and came to the conclusion that the so-called paracasein mono-lactate is simply the uncombined protein, paracasein, and that the so-called paracasein di-lactate is a compound of paracasein and lactic acid (1 gram of paracasein uniting supposedly with about 0.5 cc. of $\frac{N}{10}$ acid). It may be stated here, in passing, that it was later shown by L. L. VanSlyke and D. D. VanSlyke³ that the protein casein does not unite with acids to form insoluble compounds but that the action is simply one of *adsorption*, by which more or less acid is taken from the surrounding solution and concentrated upon the surface of the solid particles of protein; in other words, it was shown that casein or paracasein mono-lac-

¹ *Amer. Chem. Journ.*, xxviii, p. 411, 1902.

² *Ibid.*, xxxiii, p. 461, 1905.

³ *Ibid.*, xxxviii, p. 383, 1907.

tate and di-lactate have no existence as applied to the compound in question. It still remained, therefore, to find out what the brine-soluble substance really is, and work was continued along this line by the writers.⁴ We noticed that calcium is always to be found associated with the brine-soluble substance when it is separated from the other cheese constituents by extraction with a solution of calcium-free NaCl after previous removal of all water-soluble constituents. This fact suggested the possibility that the brine-soluble substance might be a combination of paracasein and calcium, containing less calcium than had been previously found in any combination of this element with paracasein. On the basis of such a possibility, it could be explained that with the formation of increased amounts of lactic acid in cheese-making, as a result of the bacterial decomposition of milk sugar, the acid would combine with more or less of the calcium contained in calcium paracaseinate, resulting in the production of a paracaseinate containing less calcium. This suggestion was strengthened by the fact that in Camembert cheese, the brine-soluble compound is formed during certain stages of the manufacturing process but soon disappears, its formation and disappearance being explained as follows, according to Bosworth:⁵ The brine-soluble substance is at first formed in Camembert cheese, as also in the case of Cheddar cheese, but, owing to the method of making this type of cheese, more acid is allowed to form in Camembert cheese, and, as a consequence, the brine-soluble substance loses its calcium and becomes free paracasein, which is insoluble in brine solution. Therefore, in the manufacture of Camembert cheese, it is found that after the first few hours the cheese contains no brine-soluble material, and, what is also significant, all the calcium is found in the water extract. The relation between the brine-soluble substance and the calcium found in the brine extract in the two types of cheese is illustrated in Table 1.

The question necessarily suggests itself whether the calcium always found in the brine-soluble extract of cheese is not there incidentally in a mechanical state rather than in a combination with paracasein. In order to study this question, the following work was done:

⁴ Technical Bulletin No. 4, 1907, New York State Agric. Exp. Sta.

⁵ Technical Bulletin No. 5, 1907, New York State Agric. Exp. Sta.

Twenty-five grams of cheese were ground with sand and extracted with water at about 55°C., using 150 cc. portions until the extract amounted to 1000 cc. The residue, containing the brine-soluble substance, was placed in a dialyzing apparatus and allowed to dialyze to insure the removal of all soluble calcium. Sodium chloride was then added to the contents of the dialyzing tube, which was then placed in a beaker of water and allowed to remain four hours. Upon adding ammonium oxalate to some of the water in the beaker, a precipitate of calcium oxalate appeared. This result leads to the belief that the Ca is present in combination in an insoluble form and that an interchange takes place between it and Na, when the insoluble compound is treated with NaCl solution.

TABLE I.

Comparison of changes in Cheddar and Camembert cheese.

AGE OF CHEESE	KIND OF CHEESE	TOTAL NITROGEN IN THE FORM OF BRINE-SOLUBLE COMPOUND	PER CENT OF TOTAL CALCIUM FOUND IN BRINE-SOLUBLE COMPOUND
		<i>per cent</i>	
When curd was cut.....	Cheddar	3.13	trace
When curd was cut.....	Camembert	6.72	trace
Ten hours.....	Cheddar	96.00	27.96
Ten hours.....	Camembert	94.00	17.76
Two days.....	Cheddar	68.87	24.47
Two days.....	Camembert	4.39	trace
Four months.....	Cheddar	43.09	24.28

In order to throw further light on the character of the brine-soluble compound, a study was made of the solvent effect of several different chlorides. One kilogram of Cheddar cheese was ground fine, thoroughly mixed, and then 25-gram portions were ground with sand, placed in bottles and extracted with water in the manner described in the preceding paragraph. The residues were then extracted with solutions of chlorides and the results given in the following table were obtained. The solutions of the salts were used in such strengths that 1000 cc. contained equivalent gram molecules. In the case of the weakest solution, extraction was continued as long as appreciable amounts of protein were obtained in the extract, 4000 cc. being used; the results in these cases are given for each 1000 cc. of extract as well as for the total.

TABLE II.

Solvent effect of neutral chlorides on the brine-soluble compound in cheese.

STRENGTH OF SOLUTION: GRAM EQUIVALENTS PER 1000 cc.	AMOUNT OF EXTRACT	PERCENTAGE OF TOTAL N IN WATER-INSOLUBLE RESIDUE OF CHEESE EXTRACTED BY					
		NaCl	NH ₄ Cl	KCl	MgCl ₂	BaCl ₂	CaCl ₂
	cc.						
1.0	1000	68.57	67.62	50.47	63.81	0	0
0.8	1000	69.29	65.24	50.47	48.33	0	0
0.6	1000	56.19	56.43	45.95	lost	0	0
0.4	1000	51.45	51.19	44.52	23.57	0	0
0.2	1st 1000	47.62	49.05	40.95	4.00	0	0
0.2	2nd 1000	13.33	10.48	13.90	5.24	0	0
0.2	3rd 1000	2.95	4.10	2.00	4.29		
0.2	4th 1000	trace	trace	trace			
Total.....	4000	63.90	63.65	56.85			

In connection with the data in the preceding table, attention is called to certain phases of the results.

1. The chlorides of Ba and Ca have no solvent effect. The chloride of Mg in strong molecular concentrations acts much like the chlorides of the alkalis, while in lower molecular concentrations its solvent power is greatly reduced.

2. Sammis and Hart⁶ attempted to study the solvent effect of these salts on the same material, but reached results not concordant with one another and not in agreement with ours. While we used solutions of such strength as to show the relation existing between the solvent action of the salt solution and its molecular concentration, they used solutions containing a uniform percentage by weight of different salts and extracted in every case with the same volume of solution. By using solutions of different salts having the same percentage composition by weight, but with a different molecular concentration, one would, under the circumstances, expect to obtain only discordant results, because the solvent effect of the solution is apparently a result of the mass action of the salt in solution, as shown by us (*this Journal*, p. 217). If Sammis and Hart had in their work continued extraction until no more solvent effect was appreciable, their results would have been in satisfactory agreement with ours. This is strikingly

⁶ *This Journal*, vi, p. 181, 1909.

shown in the above table in the case of the 0.2 N solutions; by continued extraction the total amounts extracted are found to be essentially the same as in the more concentrated solutions.

Identity of the brine-soluble compound of cheese with mono-calcium paracaseinate.

We have shown (this *Journal*, p. 223) that paracasein combines with Ca to form a compound insoluble in water but soluble in 5 per cent solution of NaCl (Na replacing Ca). In this compound we have shown that 1 gram of paracasein is in combination with 2.25×10^{-4} gram equivalents of Ca. Indications pointed to the identity of the brine-soluble substance of cheese with this mono-calcium paracaseinate, and it remained to ascertain whether the protein part of the molecule in these two compounds is the same. In order to accomplish this, a preparation of the protein in the brine-soluble compound was made from cheese, and its composition and properties were studied.

One kilogram of Cheddar cheese was ground fine and then extracted with numerous portions of distilled water at about 55°C. in order to remove all soluble compounds. The residue was then extracted with many portions of a 5 per cent solution of NaCl and filtered, first through absorbent cotton and then through paper. Dilute acetic acid was then added, giving a heavy precipitate, which was washed with water, redissolved in dilute ammonia and again precipitated with acid. The process was then completed as in the preparation of casein (this *Journal*, p. 204). The preparation on analysis gave the following results: Moisture, 2.32; ash, 0.25 per cent. In the dry substance, C, 52.97; H, 7.15; N, 15.82; P, 0.75; S, 0.78; O (by difference), 22.28.

A study of the properties of this substance gave the following results:

1. The substance is found to act as an acid in combining with bases.

2. It decomposes CaCO_3 and gives a compound in which 100 grams of substance combine with the equivalent of 2.52 grams of CaO (equal to 1.80 grams of Ca), or, 1 gram of substance combines with 9×10^{-4} gram equivalents of Ca.

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3. The solution of this calcium compound is neutral to phenolphthalein.

4. Measured by the volumetric method it was found to form a compound with ammonium represented by the combination of 1 gram of substance with 2.3×10^{-4} gram equivalents, expressed as hydroxide.

5. With Ca it forms a compound, soluble in 5 per cent solution of NaCl but insoluble in water, which contains 1 gram of substance combined with 2.3×10^{-4} gram equivalents of Ca.

6. It forms also a compound with Ca that is soluble in water, containing 1 gram of substance combined with 4.5×10^{-4} gram equivalents of Ca.

In view of the marked agreement of the composition and properties of the brine-soluble substance, formed in cheese, with the compound, mono-calcium paracaseinate, as prepared by us, there is good reason to believe that the brine-soluble substance is mono-calcium paracaseinate, having the composition of 1 gram of paracasein combined with 2.25×10^{-4} equivalents of Ca.

SUMMARY.

In many kinds of cheese there is always present a protein soluble in warm 5 per cent solution of NaCl. Previous efforts to determine the exact relation of this substance to casein or paracasein have resulted in erroneous conclusions. An extended study of its properties and composition indicates the substance to be mono-calcium paracaseinate, formed from calcium paracaseinate by removal of part of its Ca through lactic acid produced in the process of cheese making as a result of the action of lactic acid bacteria upon the milk sugar.

ON THE RATE OF EXTRACTION OF A PROTEIN (SALMINE) FROM DESICCATED TISSUE BY AN AQUEOUS SOLVENT.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, February 5, 1913.)

The testicles of the Pacific salmon (*Oncorhynchus tshawytscha*, Wahlbaum) which had been preserved and hardened in 50 per cent alcohol for two years preceding the experiment, were minced and the spermatozoa shaken out into a large bulk of distilled water. The suspension of sperm which was thus obtained was decanted from the bulk of the connective tissue and then filtered through glass wool. The spermatozoa were then agglutinated by the addition of 80 cc. of $\frac{N}{3}$ acetic acid per liter of suspension and allowed to settle out, the supernatant fluid being removed by decantation. The spermatozoa were then suspended in a volume of 95 per cent alcohol equal to the volume of the original suspension. After settling, the supernatant fluid was syphoned off and replaced by the same volume of 95 per cent alcohol. After again allowing the sperm to settle and removing the supernatant fluid, they were suspended in a volume of ether equal to one-half of the volume of the original aqueous suspension. After again allowing the sperm to settle, the supernatant ether was removed by decantation, the sperm collected in a cloth and the greater part of the ether squeezed out of them. They were then spread out upon bibulous paper to dry in the air of a warm room. The desiccated spermatozoa were thus obtained in the form of a yellowish powder containing some coarse particles. The powder was sifted through a very fine sieve, and the portion which passed through the sieve was employed in the experiment which is about to be described.

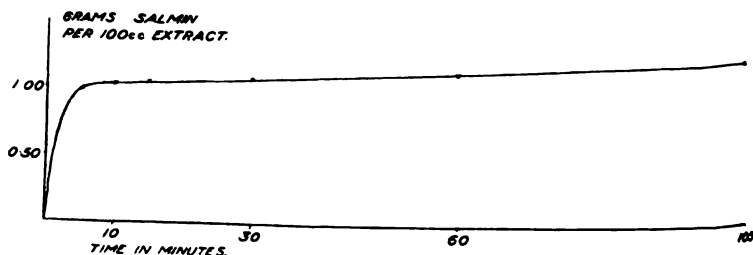
One hundred and fifty cubic centimeters of an aqueous solution of hydrochloric acid containing 1.35 per cent of HCl were placed

in a beaker, of which the diameter of the base was 7.5 cm., and agitated by a glass rod which was bent to an angle of 45° from the verticle and the tip of which, when rotated, described a circle of about 7 cm. diameter. The rod was rotated by a small motor at the very nearly constant rate of 1600 revolutions per minute. The temperature was that of the room.

Forty-five grams of the dried sperm were dropped into this fluid while stirring, and 150 cc. more of the 1.35 per cent HCl were immediately added. The entire process of dropping in the sperm and adding more fluid occupied less than fifteen seconds.

At the intervals stated below samples of the mixture were almost instantaneously withdrawn by means of a 20 cc. pipette provided with a rubber bulb. The samples were then very rapidly filtered under pressure through small dry pads of asbestos in Gooch crucibles, the filtrates being collected in dry flasks. The refractive index of the filtrate from each sample was then determined, employing a Pulfrich refractometer and a sodium flame as the source of light. Denoting the refractive index of the filtrate from any given sample by n and that of the pure solvent (1.35 per cent HCl) by n_1 , the quotient $\frac{n - n_1}{0.00172}$ is the number of grams of salmine dissolved in 100 cc. of the solvent at the moment when the sample was abstracted.¹

The relationship which was found to subsist between the period of extraction and the mass of salmine extracted is displayed graphically in the accompanying figure. It will be seen that the rate of extraction is at first very great, but that it very rapidly falls off.



It does not fall to zero, however; in other words, the curve does not approach an asymptote, in which respect it differs very strikingly

¹ T. Brailsford Robertson: *This Journal*, xi, p. 307, 1912.

from the curve which depicts the progress of a chemical reaction or of the solution of a crystalloid.

This is the type of relationship which I have previously found to subsist between the mass of casein dissolved by dilute alkali and the period during which the casein is in contact with the solvent.² It is the relationship which Cameron and Bell³ and, later, Ostwald and Goppelsroeder⁴ found to subsist between the amount of fluid absorbed by a column of sand or of a strip of filter paper and the time during which the fluid has remained in contact with a portion of its surface. This relationship may be expressed by the formula $x = Kt^m$, where x is the amount of fluid absorbed (or casein dissolved, or salmine extracted) and t is the time, K and m being constants. In the accompanying table the values of x found in the above experiment and those calculated from the formula are compared, the constants K and m being determined from all of the observations by the method of least squares, employing for this purpose the form:

$$\log_{10} x = m \log_{10} t + \log_{10} K$$

The possible experimental error in the determination of the concentration of a salmine solution by means of its refractive index (due to an error of $\pm 1'$ in reading the angle of total reflection) is ± 0.05 gram per 100 cc. It will be seen that the differences ($=\Delta$) between the observed and calculated values of x are considerably less than the possible error in the determination of the concentration of the salmine in the filtrates.

The extreme rapidity with which the salmine leaves the tissue in the first few moments of the extraction and the slowness with which the remaining amount is extracted, very vividly remind one of the extreme velocity with which, during the first instants, a liquid mounts a capillary tube and of the exceedingly slow "creeping" of the liquid up the sides of the tube which is still

² T. Brailsford Robertson: *Journ. of Physical Chem.*, xiv, p. 377, 1910.

³ Cameron and Bell: Bulletin No. 30, p. 50, Bureau of Soils, U. S. Dept. of Agriculture, 1905; *Journ. of Physical Chem.*, x, p. 658, 1906.

⁴ Wo. Ostwald: *Zeitschr. f. Kolloidchemie*, (2 Supplementheft), 1908; F. Goppelsroeder: *Verhandl. naturforsch. Gesellsch. zu Basel*, xix, Heft 2, 1907.

TIME IN MINUTES	GRAMS OF SALMINE EXTRACTED PER 100 CC. OF SOLVENT		Δ
	Found	Calculated from the formula: $x = 0.9055 t^{0.666}$	
5	0.98	0.99	+0.01
10	1.03	1.03	± 0.00
15	1.07	1.05	-0.02
30	1.07	1.08	+0.01
60	1.12	1.13	+0.01
105	1.17	1.18	+0.01
			$\Sigma\Delta = +0.02$

observable many hours after the rapid initial flow has ceased. Bearing in mind the fact that the formula $x = Kt^m$ expresses the time relations in such indubitably capillary phenomena as the absorption of water by soils or filter-paper, we may, I think, conclude that the forces which determine the rate of extraction of salmine from dried spermatozoa are capillary forces. At the same time we may infer that the accompanying *chemical* phenomena (decomposition of compounds of salmine within the tissue, formation of salmine chloride, etc.) take place at a relatively very great velocity, so that they do not appreciably affect the rate of the extraction.

Whether the capillary forces affect the rate of extraction through determining the rate of penetration of the tissue by the solvent or through determining the rate at which the dissolved salmine issues from the tissue particles, these experiments do not enable us to decide.

SUMMARY.

The rate of extraction of salmine from dried spermatozoa by dilute acid may be expressed by the formula, $x = Kt^m$, in which x signifies the amount of salmine extracted, t , the time occupied in extraction and m and K are constants.

The rate of extraction of salmine from dried spermatozoa by dilute acid is determined by capillary forces. The accompanying chemical phenomena (decomposition of compounds of salmine within the tissue, formation of salmine chloride, etc.) occur at a relatively very great velocity and hence do not affect the rate of extraction.

A QUANTITATIVE CHEMICAL ANALYSIS OF HUMAN BILE.¹

BY JACOB ROSENBLOOM.

(From the Laboratory of Biochemistry of the University of Pittsburgh, Pittsburgh, Pa.)

(Received for publication, February 10, 1913.)

Through the kindness of Dr. William Weinberger of the Lebanon Hospital, New York, there was placed at the disposal of the writer 3180 cc. of bile obtained from a patient with a biliary fistula. Since there are very few analyses of perfectly fresh human bile on record it was thought that an analysis of this fluid by modern methods would prove of interest.

One liter of the bile was treated with calcium oxide² and evaporated to dryness at 40°C. This material was powdered and placed *in vacuo* over sulphuric acid until of constant weight. Portions were then weighed out for estimation of the lipins. This was carried out according to methods described in former papers.³ The cholesterol and cholesterol esters were estimated by the excellent method of Windaus. The other constituents were estimated according to the methods described in Hoppe-Seyler's Handbuch (1909, p. 712).

The bile was preserved during the collection by the addition of a few cubic centimeters of toluene. It had all the appearances of normal bile, with a specific gravity of 1.020.

The accompanying table contains a summary of the data from various analyses of supposedly normal human bile that have appeared in the literature, together with the results obtained in this study.

¹ Some of the data contained in this paper were obtained by the writer in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons.

² This was added to form an insoluble compound with the bile pigments, which otherwise would have appeared in the ether and alcohol extracts.

³ Hanes and Rosenbloom: *Journ. of Exp. Med.*, xiii, p. 355, 1911; Rosenbloom: this *Journal*, xiii, p. 511, 1913.

Analysis
Results are expressed

OBSERVERS.....	FRIEDRICH ⁴		GORUP-BESANKE ⁵		NOTTS-SETTLER ⁶
Source.....	Bladder bile		Bladder bile		Bladder bile
Bile salts.....	72.2	91.4	107.9	56.5	39.0
Mucin and pigment.....	26.6	29.8	22.1	14.5	12.9
Cholesterol.....	1.6	2.6	47.3	30.9	3.5
Fat.....	3.2	9.2			7.3
Soaps.....					13.9
Lecithin.....					5.3
Total solids.....	140.0	148.0	177.3	101.9	
Inorganic.....	6.5	7.7	10.8	6.3	37.6
Water.....	860.0	859.2	822.7	898.1	
Fatty acids.....					

OBSERVERS.....	COPEMAN & WINSTON ¹²	YEO & HERBORN ¹³	ROBSON ¹⁴	PATON & BALFOUR ¹⁵	
Source.....	Biliary fistula	Biliary fistula (cancer)	Biliary fistula	Biliary fistula	
Bile salts.....	6.3	2.2	7.6	4.0	3.5
Mucin and pigments.....	2.5	1.5	1.3	7.1	4.6
Cholesterol.....	1.0	0.38	0.45	0.53	0.75
Fat.....			0.12	0.09	
Soaps.....			0.97	0.15	
Lecithin.....					
Total solids.....	14.23	12.8	18.0	11.9	15.3
Inorganic.....	4.5	8.8	7.6		6.4
Water.....	985.6	987.2	981.9	988.0	984.8
Fatty acids.....					

* Six analyses of bladder bile from children of the following ages: 1 day; 1 month; 2 months; 5 months; 9 months; 1 year. Figures in this column represent minimum and maximum values.

† Includes lecithin and fatty acids.

⁴ *Ann. f. d. ges. Heilk.* v, p. 42, 1845.

⁵ *Vierteljahreschr. f. prakt. Pharmakol.*, iii, p. 86, 1851.

⁶ Hoppe-Seyler: *Physiologische Chemie*, 1877-1881, pp. 299 and 301.

¹² *Journ. of Physiol.* x, p. 213, 1889.

¹³ *Ibid.*, v, p. 116, 1884.

¹⁴ *Proc. of Roy. Soc.*, xlvii, p. 499, 1890.

¹⁵ *Laboratory Reports of Royal College of Physicians, Edinburgh*, iii, p. 191, 1891.

bile.
r 1000 (by weight).

JACOBSEN ⁷	TRIFANOWSKI ⁸		HAMMARSTEN ⁹			BAGINSKY & SOMMERFELD ¹⁰	BIRCH & SPONG ¹¹
Bladder bile	Bladder bile: various diseases	Bladder bile	Liver bile			Bladder bile children	Bladder bile
10.1	28.0	19.6	9.31	18.2	9.04	25.2	0
2.3	24.8	13.0	5.29	4.29	5.15	20.0	12.1
0.6	2.5	3.3	0.63	1.6	1.5	3.4	
0.1	5.2†	3.6	0.22†	0.57	0.65	6.7	
1.4	8.2	16.3	1.23†	1.36†	1.01†		•
0.05		0.17		0.57	0.65		
22.6	91.2	89.2	25.2	35.3	25.4	103.5	20.3
5.78						9.1	8.2
977.4	909.0	911.0	974.8	964.7	974.6	896.5	979.7

HAMMARSTEN ⁹		SEBROWSKI ¹⁴	MAJEWSKI & SEBROWSKI ¹⁷	BONNANI ¹⁸	JACOBOWITZ ¹⁹	MENNIES ²⁰	ROSEN-BLOOM
Bladder bile		Biliary fistula	Bladder bile	Biliary fistula	Bladder bile (infants)*	Biliary fistula	Biliary fistula
97.0	87.0	34.3	167.6	18.33	5.5-14.0	4.2	10.1
41.9	44.4	11.14	23.12	4.97	9.0-36.0	9.3	4.86
9.86	8.7	} 5.16†	} 21.8†	1.67	1.7-3.0	0.94	2.61‡
1.9	6.5			0.96	2.5-9.8†	2.98†	6.85
11.2§	10.6§						2.6
2.23	1.41		24.7	0.58			6.42
170.3	160.2	112.2		35.44	98-145	22.5	29.8
		61.3	88.8	7.2	5.2-7.3	5.8	9.2
829.7	839.8	887.8		964.6	855-900	974.5	970.2
				1.37	0.7-1.0		1.2

† Fatty acids from soaps.

§ Includes fatty acids.

¶ This specimen contained a trace of cholesterol esters.

⁷ *Ber. d. deutsch. chem. Gesellsch.*, vi, p. 1026, 1873.

⁸ *Pflüger's Archiv*, ix, p. 492, 1874.

⁹ *Textbook of Physiological Chemistry*, 1911, p. 413.

¹⁰ *Verhandl. d. physiol. Gesellsch. zu Berlin*, 1894-95; *Arch. f. Physiol.*, 1895, p. 562.

¹¹ *Journ. of Physiol.*, viii, p. 378, 1887.

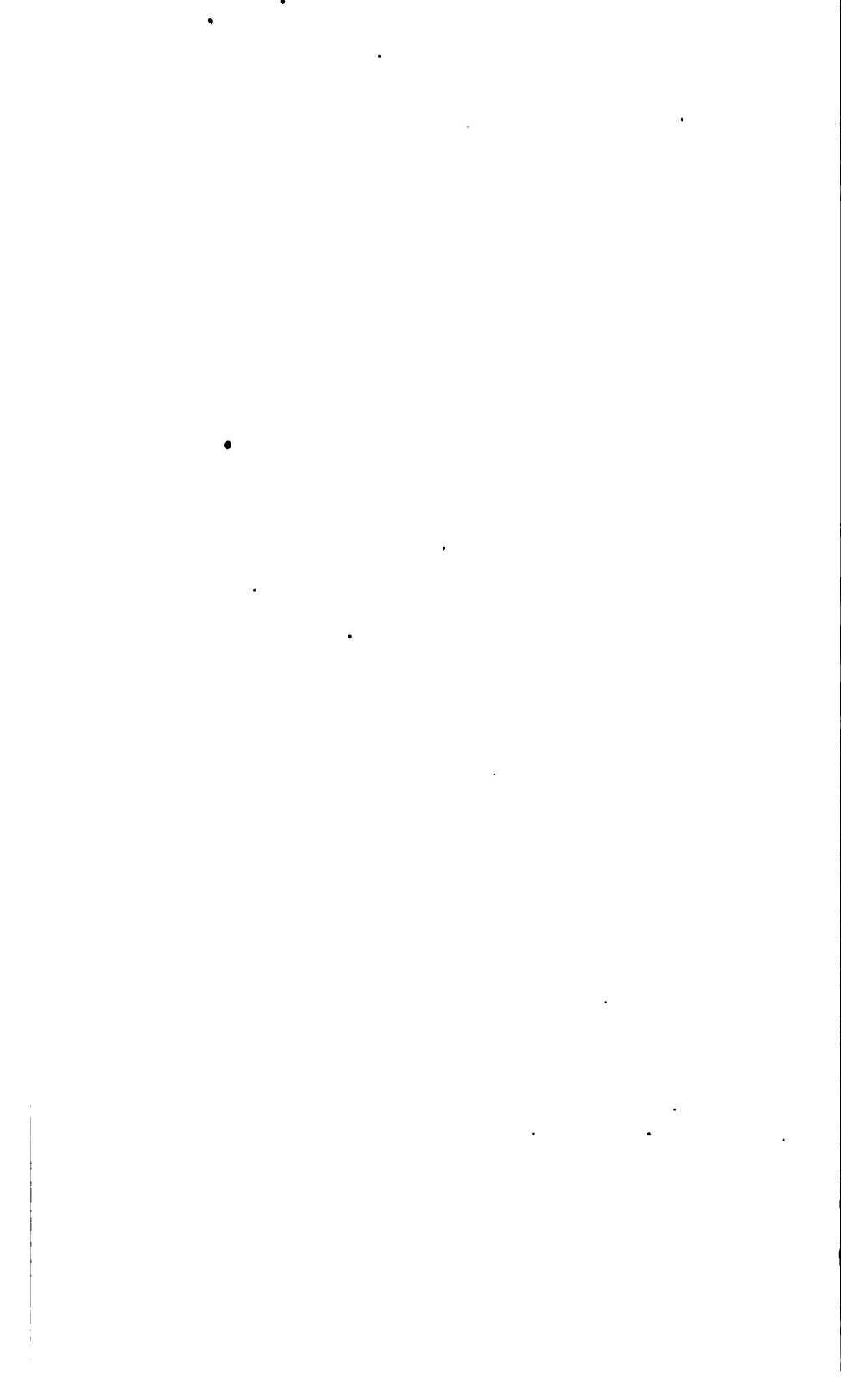
¹² *Maly's Jahresbericht*, xxxi, p. 546, 1901.

¹³ *Ibid.*, xxxii, p. 505, 1902.

¹⁴ *Ibid.*, xxxii, p. 508, 1902.

¹⁵ *Jahrb. f. Kinderheilk.*, xxiv, p. 373, 1886.

¹⁶ *Biochem. Journ.*, vi, p. 210, 1912.



THE BEHAVIOR OF SOME HYDANTOIN DERIVATIVES IN METABOLISM. II.

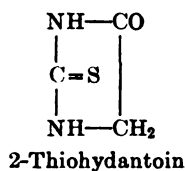
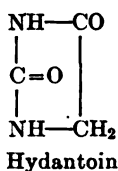
2-THIOHYDANTOINS.

By HOWARD B. LEWIS.

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(Received for publication, February 11, 1913.)

In a former paper,¹ it has been shown that the hydantoin nucleus is excreted unchanged in the urine, when introduced into the organism of the cat, rabbit or dog. The present study deals with the behavior of some thiohydantoin in which sulphur replaces the oxygen in the 2-position of the hydantoin nucleus.



The behavior of compounds containing this type of sulphur linkage is especially interesting in view of the recent studies of Johnson and his co-workers.² In these the probable existence of sulphur in the protein molecule in forms other than as cystine or cysteine groupings is discussed, and the possible occurrence of thioamide sulphur, $-\text{NHCS}-$, corresponding to the acid amide form, $-\text{NHCO}-$, present in polypeptides is suggested. This type of replacement of oxygen by bivalent sulphur is well represented in the thiohydantoin. It must be pointed out, however, that the $-\text{CS}-$ group attached to two nitrogen atoms as in thiohydantoin is much

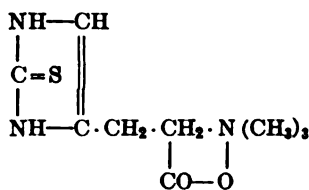
¹ This *Journal*, xiii, pp. 347-56, 1912.

² Johnson: this *Journal*, ix, pp. 331-2, 439-48, 449-63, 1911; xii, pp. 175-96, 1912.

more stable than the $-CS-$ group placed between a nitrogen and a carbon atom as in a thioamide, $R-CS-NH_2$, or as in dithiopiperazine, the anhydride of the thiopolypeptide, recently prepared by Johnson and Burnham.³ In the latter compound the sulphur may be readily split off as hydrogen sulphide by boiling with hydrochloric acid.

Since thiohydantoin contains the thioamide type of sulphur grouping, a study of the ability of the organism to oxidize and excrete the sulphur of these compounds should throw some light on the possible behavior of similar sulphur groupings, whose presence in the protein molecule has been suggested.

It has recently been shown that a compound which closely resembles the thiohydantoin may be obtained from the ergot of rye. Barger and Ewins⁴ have found that the base *ergothioneine* isolated from ergot by Tanret⁵ is probably the betaine of α -amino- β -2-thioglyoxaline-4 (or 5)-propionic acid.



Ergothioneine

The simplest compound which contains this type of sulphur combination is thiourea, $NH_2-CS-NH_2$, the behavior of which has repeatedly been the subject of study.⁶ Thiourea is non-toxic, is excreted unchanged in the urine, and does not increase the oxidized sulphur content of the urine. After the administration of thiourea to rabbits, Pohl⁷ reports the elimination of an alkyl

³ Johnson and Burnham: this *Journal*, ix, pp. 449-63, 1911.

⁴ Barger and Ewins: *Journ. of Chem. Soc. (London)*, xcix, pp. 2336-41, 1911.

⁵ Tanret: *Journ. de pharm. et de chim.*, xxx, pp. 145-53, 1909; *Compt. rend. de l'Acad. des Sci.*, cxlix, pp. 222-24, 1909.

⁶ Cf. Lange: Inaugural Dissertation, Rostock, 1892; *Jahresber. u. d. Fortsch. d. Thierchem.*, xxii, p. 67, 1892; Sato: *Zeitschr. f. physiol. Chem.*, lxxiii, pp. 378-96, 1909; Masuda: *ibid.*, lxxvii, p. 28, 1910.

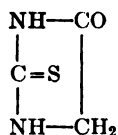
⁷ Pohl: *Arch. f. exp. Path. u. Pharm.*, li, pp. 341-45, 1904.

sulphide, probably ethyl sulphide, in the breath. No sulphide could be detected in the urine. The greater part of the thiourea was excreted unchanged and only a few milligrams of alkyl sulphide were obtained from the expired air.

In the present work, two preliminary experiments in which 2 grams of thiourea were injected subcutaneously into rabbits were made. In confirmation of the work of Pohl, a peculiar garlic-like odor was observed in the breath five hours after the injection, but no alkyl sulphide could be obtained from the urines by the method of Abel.⁸ The total sulphate-sulphur content of the urine was unchanged, while the "neutral sulphur" was increased in proportion to the amount of sulphur injected as thiourea. The urines of the experimental days gave strong reactions with potassium ferrocyanide and acetic acid, as described for thiourea by Sato.⁹ No toxic effects were apparent.

In the experiments to be recorded with the thiohydantoins the animals used were, with one exception, rabbits, which were maintained on a uniform diet of carrots and oats. The urine was collected from the bladder by gentle pressure at the same hour daily. The substances, when fed, were dissolved in water and introduced through a stomach tube. The routine analytical procedures included the Kjeldahl-Gunning method for total nitrogen, Folin's method for total sulphate-sulphur, and Benedict's methods¹⁰ for urea and total sulphur. "Neutral sulphur" was obtained by difference. It is of interest to note that all of the compounds studied give the color reaction with phosphotungstic acid and sodium carbonate described by Folin for uric acid.

2-Thiohydantoin.



⁸ Abel: *Zeitschr. f. physiol. Chem.*, xx, pp. 253-78, 1895.

⁹ Sato: *Biochem. Zeitschr.*, xxiii, pp. 45-6, 1910.

¹⁰ S. R. Benedict: this *Journal*, vi, pp. 363-71, 1909 (total sulphur); *ibid.*, viii, pp. 405-22, 1910 (urea).

This compound was prepared from hippuric acid and ammonium thiocyanate as described by Johnson and Nicolet.¹¹ Its purity was established by its melting point (226–7°) and a Kjeldahl nitrogen determination (24.12 per cent N). The compound had a slight red-yellow color and when dissolved in water gave a yellowish solution. With picric acid and an alkali a color resembling that given by creatinine in Jaffé's test was obtained.

The substance proved to be toxic for rabbits, doses of 0.5 gram causing death within twelve hours. The most noticeable symptoms were loss of muscular control and power of co-ordination, dyspnoea and convulsions. The degree of toxicity is shown by the following illustrative protocol and the accompanying table (I) which summarizes all the results obtained.

Rabbit 14. Weight 1.4 kgms. 8.30 a.m. Received 0.5 gram of 2-thiohydantoin subcutaneously. Resumed eating on return to cage.

9.00 Has stopped eating.

10.00 Apparently normal.

11.00 Apparently normal except for slightly increased respiration.

12.00 Restless. Poor control of hind legs. Tremors. Rapid respiration.

1.00 Lack of coördination. Control of hind legs lost. Dyspnoea.

2.00 An occasional convulsion.

2.55 More quiet. Convulsions less frequent. Dyspnoea.

3.10 Urinates. Urine deep orange red.

3.45 Violent convulsions, dyspnoea. Pupils very greatly dilated.

4.00 Dead.

Autopsy. Muscles stained yellow red at point of injection. Fluid all absorbed. Viscera appear normal. Bladder empty. Lungs and heart congested.

Examination of Urine. Color deep orange red. Jaffé's picric acid test very brilliant. Albumin test negative. Reduction test with Benedict's solution gives a black precipitate of copper sulphide.

¹¹ Johnson and Nicolet: *Journ. Amer. Chem. Soc.*, xxxiii, p. 1973, 1911; Johnson: *Amer. Chem. Journ.*, xlix, pp. 68–9, 1913.*

TABLE I.

Toxicity of 2-Thiohydantoin.

ANIMAL	WEIGHT		DOSE PER KGM.	METHOD OF ADMINISTRATION	RESULTS
	kgms.	grams			
Rabbit 12.....	1.70	1.17	<i>per os</i>		Death in 3½ hours.
Rabbit 13.....	1.67	0.59	<i>per os</i>		Death in 4½ hours.
Rabbit 14.....	1.40	0.31	subcutaneously		Death in 7½ hours.
Rabbit 15.....	1.58	0.125	subcutaneously		Death in 24 hours.
Rabbit 16.....	1.48	0.066	subcutaneously		Refused food for 2 days. Restless. Recovered.
Rabbit 19.....	1.80	0.277	subcutaneously		Preparation 2.* Death in 7½ hours.
Rabbit 22.....	1.92	0.260	subcutaneously		Preparation 3.* Death 9½ hours.
Rabbit 27.....	1.78	0.056	subcutaneously		Recovery. Refuses food for two days.
Rabbit 28.....	1.63	0.061	subcutaneously		Recovery. Refuses food for two days.
Rabbit 29.....	1.70	0.088	subcutaneously		Recovery. Refuses food for two days.
Cat C.....	3.50	0.157	<i>per os</i>		Death in 8 hours.

* After the earlier experiments had shown the marked toxicity, a new preparation 2 was prepared and especially purified. Preparation 3 was a preparation used in experiments with rabbits 19, 22, 27 and 29, and cat C, especially purified by Mr. Ben H. Nicolet to whom I am indebted for this and other assistance.

The toxicity of this compound must be attributed to the sulphur which replaces the oxygen in the hydantoin nucleus. While approximately 0.125 gram per kilo body weight is the lethal dose for the thiohydantoins, amounts of over 1.5 grams of hydantoin per kilo have been fed to rabbits without any toxic effects.

It was first planned to study the distribution of sulphur in the urine after the administration of the thiohydantoin in order to note any oxidation of the sulphur. But the relatively small size of the dose which is necessary to prevent a fatal outcome of the experiment made such a study unpromising. An attempt was made in the case of rabbits 22, 27, 28, 29, and cat C to identify the unchanged thiohydantoin in the urine.

Many attempts to isolate the thiohydantoin as such were unsuccessful. At length a method of procedure was adopted which, while it gave positive evidence of the presence of unchanged thiohydantoin in the urine, did not furnish absolute proof. Thio-

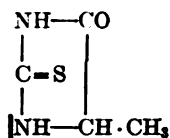
hydantoin when boiled with chloroacetic acid are desulphurized¹² with the formation of hydantoin and thioglycollic acid. The latter acid may be detected by a delicate color reaction described by Heffter.¹³ A very dilute solution of thioglycollic acid gives, on the addition of sodium nitroprusside and an alkali, a purple-red color, changing quickly to brown-red and soon disappearing. This reaction is also given by the alkyl sulphides, ethyl and benzyl mercaptans, cysteine, α - and β -thiolactic acids and thiophenol.

The urines were first tested for this reaction before treatment with chloroacetic acid to rule out the presence of the alkali sulphides and the other above-mentioned compounds which give this reaction. In no case was any purple color obtained from the dilute urine. The urine was then concentrated on the water bath to small volume, 2-3 grams of chloroacetic acid added, and the mixture boiled with a return condenser from four to six hours. The contents of the flask were cooled, treated with animal charcoal to decolorize, and the color reaction carried out. In all the experimental urines the reaction was positive. Normal urines which were treated in the same manner never gave positive reactions. To avoid the color of the urine, which interfered with a delicate test, the liquid after boiling with chloroacetic acid was in some cases evaporated to dryness and extracted with ether, in which thioglycollic acid is soluble. The ether was then removed by evaporation, the residue taken up in water, and the color reaction carried out. The reactions were more brilliant when the test was performed in this way.

While the possibility that there may be other compounds formed in the body, which yield thioglycollic acid on boiling with chloroacetic acid, is not entirely excluded, the demonstration of the presence of thioglycollic acid after the chloroacetic acid treatment in the experimental urines gives a strong indication of the presence of unchanged thiohydantoin. 2-Thiohydantoin, like hydantoin, is eliminated probably unchanged by the rabbit. Unlike hydantoin it is very toxic for rabbits, 0.125 gram per kilo being a lethal dose.

¹² Johnson, Pfau and Hodge: *Journ. Amer. Chem. Soc.*, xxxiv, pp. 1041-48, 1912.

¹³ Heffter: *Medizin.-naturwiss. Archiv*, i, p. 81, 1908.

2-Thio-4-methylhydantoin.

2-Thio-4-methylhydantoin was prepared by the action of ammonium thiocyanate on alanine as described by Johnson.¹⁴ Its purity was shown by its melting point (161°) and a Kjeldahl nitrogen determination (21.53 per cent N).

2-Thio-4-methylhydantoin was toxic for rabbits although far less so than the 2-thiohydantoin. The lethal dose was 0.6 gram per kilo body weight, or more than three times that of 2-thiohydantoin. The symptoms were very similar to those produced by the 2-thiohydantoin, although the convulsions were less severe than with the latter. Most marked was the intense albuminuria in all those cases in which the fatal dose was given. A study of the typical protocol given below, together with the tabular summary (table II), will best serve to illustrate the toxicity.

Rabbit 34. Weight 1.7 kilos. Dec. 12, 8.30 a.m. Received 1 gram of 2-thio-4-methylhydantoin subcutaneously.

11.00 Drowsy. Sits with eyes closed.

12.00 Tremors. Runs excitedly around cage when roused.

3.00 Very excitable. Runs around cage. Occasional convulsions.

5.00 Quiet.

December 13. 8.00 a.m. Drowsy. Urine deep yellow. Protein tests positive (Heller's, picric acid, heat coagulation). No casts.

Animal quiet all day. Ate no food.

Dec. 14. 8.00 a.m. Animal dead in cage. Body still warm. Urine, protein test strongly positive.

Autopsy revealed nothing abnormal.

¹⁴ Johnson: this *Journal*, xi, pp. 97-101, 1912; *Amer. Chem. Journ.*, xlix, pp. 68-9, 1913.

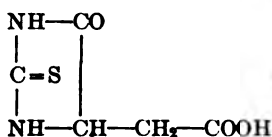
TABLE II.

Toxicity of 2-Thio-4-Methylhydantoin.

ANIMAL	WEIGHT	DOSE PER KGM.	METHOD OF ADMINISTRATION	RESULTS
	<i>kgs.</i>	<i>grams</i>		
Rabbit 20.....	2.1	0.25	<i>per os</i>	No symptoms.
Rabbit 21.....	1.9	0.25	subcutaneously	No symptoms.
Rabbit 20.....	2.1	0.50	<i>per os</i>	Restless. Refuses food for two days.
Rabbit 31.....	1.6	0.93	subcutaneously	Death in 31 hours. Al- bumin test positive.
Rabbit 33.....	1.2	0.83	subcutaneously	Death in 1½ hours.
Rabbit 34.....	1.7	0.58	subcutaneously	Death in 2 days. Al- bumin test positive.
Rabbit 35.....	1.8	0.69	<i>per os</i>	Death in 5½ hours. Al- bumin test positive.
Rabbit 36.....	1.7	0.58	<i>per os</i>	Death in 3 days. Al- bumin test negative.

No attempt was made to identify the unchanged thiohydantoin in the urine.

The chief interest in the above experiments lies in the lowered toxicity of the sulphur hydantoins due to the substitution of a methyl group for one hydrogen in the 4-position.

2-Thiohydantoin-4-acetic acid.

This hydantoin was prepared from ammonium thiocyanate and asparagine as described by Johnson and Guest.¹⁵ A determination of its melting point (222°) and nitrogen content (16.09 per cent N) showed its purity. This compound is rather difficultly soluble in water; hence in the experiments in which it was used sodium carbonate was added to form the sodium salt which is more soluble.

¹⁵ Johnson and Guest: *Amer. Chem. Journ.*, xlviii, pp. 108-9, 1912; Johnson: *ibid.*, xlix, pp. 68-9, 1913.

After several preliminary experiments had shown that 2-thiohydantoin-4-acetic acid was non-toxic for rabbits and that as large a dose as 2 grams had no obvious effects on the animal, a study of the sulphur elimination and distribution in the urine was made.

Rabbit 30. Daily diet, 250 grams of carrots and 25 grams of oats. This was completely consumed. On the day of the injection the animal showed no symptoms, except a refusal to eat for several hours. On the fourth day, a subcutaneous injection of 2 grams of 2-thiohydantoin-4-acetic acid was made (N content = 0.321 gram, S content = 0.368 gram). The protocol follows.

Rabbit 30; Weight, 1.69 kgms.

VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA +NH ₂ -N	UREA +NH ₂ -N	N NOT UREA +NH ₂ -N	TOTAL S	TOTAL SO ₄ S	NEUTRAL S	TOTAL SO ₄ S	NEUTRAL S
				Total N						
cc.		gram	gram	per cent	gram	gram	gram	gram	per cent	per cent
85	1.040	0.675	0.555	82.2	0.120	0.0667	0.0575	0.0092	86.2	13.8
125	1.020	0.720	0.615	85.4	0.105	0.0642	0.0536	0.0106	81.9	18.1
125	1.030	0.570	0.465	81.7	0.105	0.0629	0.0497	0.0132	79.0	21.0
100	1.040	1.088	0.735	67.5	0.353	0.3478	0.0306	0.3172	8.8	91.2
125	1.023	0.705	0.570	80.8	0.135	0.0567	0.0371	0.0196	65.4	34.6

Rabbit 40. Diet, 25 grams of oats and 250 grams of carrots. This diet was consumed as usual on the day of the administration of the 2-thiohydantoin-4-acetic acid. On the fourth day 1.75 grams of 2-thiohydantoin-4-acetic acid were given *per os* (S content = 0.322 gm.) No toxic symptoms were noted. The protocol follows.

Rabbit 40; Weight, 1.82 kgms.

DATE	VOLUME	SPECIFIC GRAVITY	TOTAL S	TOTAL SO ₄ S	NEUTRAL S	SO ₄ S	NEUTRAL S
	cc.		gram	gram	gram	per cent	per cent
1	100	1.015	0.0718	0.0561	0.0157	78.1	21.9
2	180	1.014	0.0767	0.0566	0.0201	74.8	26.2
3	150	1.020	0.1167	0.0809	0.0358	69.3	30.7
4	160	1.023	0.2824	0.0681	0.2244	20.5	79.5
5	80	1.036	0.1596	0.0790	0.0806	49.5	50.5
6	160	1.015	0.0980	0.0691	0.0289	70.5	29.5
7	160	1.019	0.0946	0.0728	0.0218	77.0	23.0

In a third experiment in which 1.75 grams of 2-thiohydantoin-4-acetic acid were given *per os* to a rabbit of 1.5 kilos, similar results were obtained, the "neutral sulphur" rising from a preliminary average of 23.2 per cent to 88.8 per cent on the day of the injection.

In these three experiments on rabbits there was no evidence of an oxidation of the sulphur by the organism, a reaction which should result in an increased total sulphate-sulphur elimination. In no case was the sulphate-sulphur increased on the day of the injection. In each instance, however, the "neutral sulphur" elimination was increased in proportion to the amount of sulphur given as 2-thiohydantoin-4-acetic acid. No attempt was made to recover the unchanged hydantoin from the urine.

It is interesting to note that these results, which show that the sulphur in this type of combination is not oxidized by the organism, are in agreement with the results obtained by Steudel¹⁶ and Mendel and Myers.¹⁷ The former working with 2-thio-4-methyluracil found that it was excreted unchanged by the organism of the dog. The latter studied the distribution of sulphur in the urine after the administration of 2-thiouracil to rabbits and found no increase in the oxidized sulphur, but a marked increase in the "neutral sulphur" of the urine. These findings all agree in demonstrating the stability of thioamide sulphur in the organism.

Experiments on rabbits in which doses of 0.44 and 0.79 gram per kilo of 2-thiohydantoin-4-acetamide were given *per os* to rabbits demonstrated that in such amounts this substance is non-toxic for rabbits.

The lack of toxicity of 2-thiohydantoin-4-acetic acid for the rabbit would seem to indicate that the substitution of alkyl groups or their oxidation products for a hydrogen in the 4-position diminishes the toxicity shown by the sulphur in 2-thiohydantoin. The increase in the molecular weight of the substituted alkyl group, in the substances studied at least, gradually decreases the toxicity. Thus the lethal dose of the unsubstituted thiohydantoin was found to be about 0.125 gram, the substitution of a methyl group raised the fatal dose to 0.6 gram, while substitution of an acetic acid or acetamide group caused a loss of toxicity. It is of interest to know whether this theory of decreased toxicity will be confirmed by a

¹⁶ Steudel: *Zeitschr. f. physiol. Chem.*, xxxix, pp. 136-42, 1903.

¹⁷ Mendel and Myers: *Amer. Journ. of Physiol.*, xxvi, pp. 77-105, 1910.

study of the effect of other radicals, substituted both in the 4-position as in the compounds studied and in other positions in the ring. An investigation of the effects of such substitution is proposed.

SUMMARY.

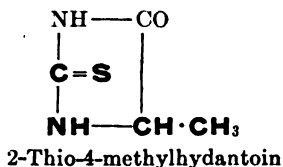
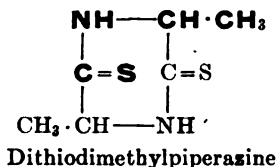
2-Thiohydantoin is toxic for rabbits. The substitution of an alkyl group in the 4-position decreases the toxicity. 2-Thio-4-methylhydantoin is less toxic than 2-thiohydantoin, while 2-thiohydantoin-4-acetic acid is not toxic in doses of 2 grams. 2-Thio-4-methylhydantoin in fatal doses causes an albuminuria in rabbits. The sulphur contained in 2-thiohydantoins is not oxidized in the organism of the rabbit, but is excreted probably unchanged.

I wish to acknowledge my indebtedness to Professor Lafayette B. Mendel under whose direction this work has been carried out and to Professor Treat B. Johnson who has aided in the questions of organic chemistry involved.

ADDENDUM.

Through the courtesy of Professor Treat B. Johnson, a study of one of the thiopolypeptides already referred to was made possible. The compound with which the experiments were carried out was *dithiodimethylpiperazine*, the preparation and properties of which will be described in a later paper from Professor Johnson's laboratory. The new compound is very insoluble in water and was administered as the sodium salt. It was first suspended in water, sodium hydroxide added in the cold until solution took place, and the whole immediately neutralized. These precautions were made necessary because of the ease with which dithiodimethylpiperazine splits off sulphur in the presence of free alkali. As has already been pointed out, this thioamide type of sulphur combination is very unstable.

The relation of this thiopolypeptide derivative to the thiohydantoins which have been shown to be toxic may be seen from a comparison of the structural formulae.



Two $-\text{CS.NH.CH.CH}_3$ groupings make up the thiopolyptide derivative. This same grouping occurs once in the thiohydantoin. Hence from a theoretical standpoint, the toxicity of the thiopiperazine derivative should be greater than that of the thiohydantoin. This was found to be the case. Two typical protocols follow.

Rabbit 43. Weight 1.70 kgms. 10.07 a.m. Received 0.3 gram (= 0.176 gram per kilogram body weight) dithiodimethylpiperazine, prepared as described above, *subcutaneously*.

10.25 Tremors. Very restless. Dyspnoea.

10.30 Slight convulsions.

10.35 Dyspnoea very marked. Animal gasps, gnashes teeth.

10.40 Tremors more marked. Pupils widely dilated.

10.45 Animal has a very violent convulsion in the course of which it forces open the cover of the cage. Immediately following tetanic symptoms, not clonic, appear.

10.55 Convulsions very frequent. As animal was apparently dying ether was given to neutralize the effects of convulsions. Animal became quiet immediately and on being allowed to recover from the anesthesia showed convulsions again.

12.05 The animal had been etherized for more than an hour, but the increasing violence and frequency of the convulsions made deeper anesthesia constantly necessary. The animal was now allowed to recover from anesthesia. Immediately, extreme dyspnoea, labored breathing and convulsions began.

12.35 Very weak.

1.15 Dead.

Autopsy. Nothing abnormal. Urine: trace of albumin.

Rabbit 45. Weight 1.8 kgms. 9.00 a.m. Received 0.3 gram dithiodimethylpiperazine prepared as described above *per os*.

9.07 Tremors.

9.10 Convulsions.

9.15 Lies on side in convulsions.

9.23 Extreme dyspnoea.

9.33 Very weak.

10.00 Death.

Autopsy. Nothing abnormal except congested heart and lungs.

The close parallelism between the symptoms above described and those produced by alkali sulphides or hydrogen sulphide immediately suggests that an explanation of the toxicity may be found in the liberation of sulphides in the organism from the unstable sulphur linkage. Further investigations on this substance are in progress.

ON CEREBRONIC ACID

SECOND PAPER.

By P. A. LEVENE AND C. J. WEST.

(*From the Rockefeller Institute for Medical Research, New York.*)

(Received for publication, February 19, 1913.)

On the basis of experiments published in a previous communication Levene and Jacobs¹ reached the view that cerebronic acid had the structure of an α -hydroxypentacosanic acid. On reduction the acid formed a hydrocarbon melting at 54°–57°C. The melting point for the normal pentacosan, according to Krafft and Marie, should be 53.5°–54°. Hence it remained uncertain whether the discrepancy in the melting points was due to the fact that the hydrocarbon obtained from cerebronic acid contained some impurity or to a structural difference in the normal pentacosan and in the hydrocarbon obtained from cerebronic acid. It was, therefore, concluded to prepare a larger quantity of the material, which would permit of a more perfect purification of the hydrocarbon.

This was accomplished with the result that on reduction of cerebronic acid a hydrocarbon was obtained that melted at 53°–54°C., which harmonizes with the melting point corresponding to the normal pentacosan.

The normal nature of the carbon chain of cerebronic acid was corroborated further by the fact that the acid, $C_{24}H_{48}O_2$, obtained on the oxidation of the former was reduced to a hydrocarbon melting at 51°–52°C., and that Krafft found 51.1°C. to be the melting point of the normal tetracosan. Hence, it may be considered proven that cerebronic acid is a normal α -hydroxypentacosanic acid.

In course of the present investigation great care was taken in establishing the exact condition for preparation of the most important derivatives of the acid, also of the methods of separation and of purification of the acid.

¹ This *Journal*, xii, p. 381, 1912.

EXPERIMENTAL PART.

Preparation of cerebronic acid.

The mixture of cerebronic acid and ester obtained from the hydrolysis of cerebrine² is heated with an excess of alcoholic sodium hydroxide for four hours, during which most of the sodium salt separates out. The mixture is cooled, the soaps filtered off and washed with methyl alcohol and ether. The sodium salt is then recrystallized from boiling ethyl or methyl alcohol. The cerebronic acid is liberated by suspending the salt in dilute hydrochloric acid and heating on the water bath until the free acid has completely melted. When cold this is filtered off, recrystallized from alcohol and the excess of alcohol removed by melting in vacuum on the water bath. The product thus obtained is pure enough for most operations. It will not, however, give sharp values when titrated to determine the molecular weight. The acid used for this purpose must be further purified through the lead salt. The hot methyl alcoholic solution of the acid is treated with a hot solution of lead acetate in the same solvent as long as a precipitate forms. Finally a few drops of concentrated ammonia are added to neutralize the free acetic acid. After being cooled the lead salt is filtered off and washed with a little warm methyl alcohol. It is then suspended in warm, dry toluene and decomposed by treating with hydrogen sulphide for two hours. The mixture should be kept warm on a water bath and constantly stirred during this time. The lead sulphide is allowed to settle, the toluene solution of the acid filtered off, the sulphide washed with warm toluene and the filtrate concentrated in vacuum. The colorless residue is then recrystallized from absolute alcohol and the excess of alcohol removed on the steam bath. If ammonia is not added in the precipitation of the lead salt the sulphide may come down in a colloidal form. In this case, the entire mixture is concentrated in vacuum and the acid extracted from the residue with a large quantity of boiling alcohol.

The acid thus prepared melted at 77°–80° and, therefore, consisted principally of the inactive form. When fractionated with lithium acetate in methyl alcohol into three fractions, each fraction,

² Levene and Jacobs: *loc. cit.*, p. 383.

before recrystallization, melted at 76°–77° and, when recrystallized from petroleum ether, melted at 83°–84°. This is the melting point found for the inactive form and reported in a previous paper.

0.1200 gram of the substance gave 0.3330 gram CO₂ and 0.1350 gram H₂O.

1.7115 grams of the acid dissolved in a mixture of benzene and pure methyl alcohol required 43 cc. of $\frac{N}{10}$ NaOH for neutralization, using phenolphthalein as an indicator.

0.4802 gram of the acid, as above, required 12.15 cc. of $\frac{N}{10}$ NaOH for neutralization.

	Calculated for C ₂₄ H ₄₀ O ₇		Found:
C.....	75.33		75.68
H.....	12.50	-	12.59
M. W.....	398	398	395

Salts of cerebronic acid.

Salts. The sodium and lithium salts were prepared and their solubilities studied in order to find the best solvent for purification and also to establish the conditions for the separation of the oxidation product of cerebronic acid from the unchanged acid (see below). The determinations were carried out as follows: The salt was treated with an excess of the boiling solvent; the saturated solution was filtered off, using a hot funnel, and allowed to stand in the ice box over night. The precipitate was filtered off, dried and weighed. The residue from the filtrate was also determined. This gave the total solubility, the amount that could be obtained in recrystallization and the solubility at 0°.

Sodium salt.² SOLUBILITIES. 140 cc. of boiling methyl alcohol, saturated with the sodium salt, deposited, upon cooling to 0°, 1.47 grams. 20 cc. of the filtrate, saturated at 0°, gave a residue of 0.09 gram upon evaporation to dryness.

110 cc. of ethyl alcohol, as above, deposited 2.094 grams. 20 cc. of the filtrate gave a residue of 0.032 gram.

Therefore, 100 cc. of boiling methyl alcohol takes up 1.50 grams of the sodium salt. At 0° it contains 0.45 gram.

100 cc. of boiling ethyl alcohol contains 2.06 grams; at 0°, 0.16 gram.

² Thierfelder: *Zeitschr. f. physiol. Chem.*, xliii, p. 26, 1904-05.

ANALYSIS. 0.1125 gram of the substance gave 0.2931 gram CO_2 and 0.1196 gram H_2O .

	Calculated for $\text{C}_{25}\text{H}_{40}\text{O}_8\text{Na}$:	Found:
C.....	71.19	71.06
H.....	11.72	11.90

Lithium salt. SOLUBILITIES. 100 cc. of boiling methyl alcohol takes up 3.46 grams of the salt, while at 0° it contains 0.235 gram.

100 cc. of ethyl alcohol at its boiling point takes up 1.42 grams of the salt and at 0° contains 0.39 gram.

ANALYSIS. 0.1448 gram substance gave 0.3928 gram CO_2 and 0.1593 gram H_2O .

	Calculated for $\text{C}_{25}\text{H}_{40}\text{O}_8\text{Li}$:	Found:
C.....	74.18	74.00
H.....	12.21	12.31

Inactive ethyl ester.

Thudichum⁴ describes an ethyl ester of neurostearic acid (cerebronic acid) which he obtained from the hydrolysis of phrenosine (cerebrine) with ethyl alcohol and sulphuric acid. This product melted at 56° but when distilled in vacuum melted at 52° . Since the acid derived from this melted at 84° it must have been the inactive ester. We prepared the ester from the inactive acid as follows: 10 grams of the acid were dissolved in 500 cc. absolute ethyl alcohol, 10 cc. concentrated sulphuric acid added and the mixture heated on the water bath for seven hours. Upon standing over night at 0° the ester almost completely separates out. This was recrystallized from alcohol containing sulphuric acid, then from absolute alcohol and finally from dry acetone. It is very soluble in ethyl acetate, from which it separates in large glistening crystals. The ester melted at 52° – 53° and solidified at 51° – 52° . For analysis the product was dried three hours in a chloroform bath over sulphuric acid.

0.1128 gram substance gave 0.3142 gram CO_2 and 0.1265 gram H_2O .

	Calculated for $\text{C}_{25}\text{H}_{40}\text{O}_8\cdot\text{C}_2\text{H}_5$:	Found:
C.....	75.79	75.98
H.....	12.55	12.75

⁴ Thudichum: *Chemical Constitution of the Brain*, p. 162; German edition, p. 195.

. *Acetate of the ethyl ester*, $C_{22}H_{47}CH(OCOCH_3)CO_2C_2H_5$.

Two grams of the ethyl ester were dissolved in 40 cc. of acetic anhydride and heated to gentle boiling for an hour. Upon cooling the reaction product crystallized out. This was recrystallized from acetone and twice from petroleum ether. It forms colorless crystals, which melt at 55° – 57° and solidify at 53° – 55° . It was dried in the chloroform bath for analysis.

0.1336 gram of substance gave 0.3630 gram CO_2 and 0.1457 gram H_2O .

	Calculated for $C_{22}H_{47}O_4$	Found:
C.....	74.28	74.16
H.....	12.05	12.19

Marie⁶ describes a similar compound which was obtained by the action of lead acetate upon the ethyl ester of bromocerotic acid and to which he ascribes the above formula. This melted at 57° – 58° . Since then it has been claimed that cerotic acid contains twenty-six carbon atoms; if this is so, then the two compounds are not identical. The question will be investigated further.

Inactive methyl ester.

Thierfelder⁶ describes a methyl ester of cerebronic acid, which he obtained in the hydrolysis of cerebrine and which gave upon hydrolysis an acid melting at 100° – 101° . This ester melted at 65° , and was probably either the active form or a mixture of the active with the inactive. The inactive ester was prepared by boiling a solution of 5 grams of the inactive acid in 500 cc. of absolute methyl alcohol, which contained 5 cc. of concentrated sulphuric acid, for five hours. The ester which separated out upon standing over night was recrystallized from methyl alcohol, petroleum ether and finally acetone and formed colorless crystals, which melted at 59° – 60° and solidified at 57° – 58° . It was dried in the chloroform bath for analysis.

0.1120 gram substance gave 0.3096 gram CO_2 and 0.1281 gram H_2O .

	Calculated for $C_{26}H_{52}O_2 \cdot CH_3$	Found:
C.....	75.65	75.39
H.....	12.71	12.80

⁶ Marie: *Ann. de chim. et de phys.*, (7), vii, p. 228, 1896; *Bull. de la soc. chim.*, xv, p. 577, 1896.

⁷ *Zeitschr. f. physiol. Chem.*, xlv, p. 367, 1905.

Acetyl cerebronic acid.

Thierfelder⁷ attempted to prepare acetyl cerebronic acid by the action of acetyl chloride upon cerebronic acid; he was not able to isolate the free acid in a crystalline condition, but analyzed it as the sodium salt. By the use of acetic anhydride the reaction is smooth and the product easy of isolation.

Two grams of cerebronic acid and 30 cc. of acetic anhydride were heated under a reflux for two hours. Upon cooling the solution the acetylated acid separated out in a crystalline condition. This was filtered in the ice box, washed with cold alcohol several times to remove the acetic anhydride and twice recrystallized from petroleum ether. It is easily soluble in nearly all organic solvents. Acetyl cerebronic acid is a white crystalline solid, which melts at 555°-56° and solidifies at 53°-54°. Since the inactive acid was used, this product is the inactive form. The active form would melt higher.

ANALYSIS. 0.1144 gram of the substance gave 0.3090 gram CO₂ and 0.1240 gram H₂O.

	Calculated for C ₂₇ H ₄₈ O ₈ :	Found:
C.....	73.57	73.50
H.....	11.90	12.15

MOLECULAR WEIGHT ESTIMATION. 0.2776 gram of the substance, dissolved in pure methyl alcohol and benzene, required 6.3 cc. of $\frac{N}{10}$ NaOH for neutralization.

	Calculated for C ₂₇ H ₄₈ O ₈ :	Found:
M. W.....	440	441

ACETYL DETERMINATION. 0.0908 gram of the dried substance was dissolved in 10 cc. of sodium methylate and 50 cc. of pure methyl alcohol, equal to 113.6 cc. of $\frac{N}{10}$ HCl, and heated under the reflux for an hour. The reaction product then required 109.8 cc. of $\frac{N}{10}$ HCl for neutralization. Thus, 3.8 cc. of $\frac{N}{10}$ NaOH were required in the determination. The amount calculated for the splitting off of one acetyl group and the neutralization of the cerebronic acid formed in the reaction is 4.01 cc. of $\frac{N}{10}$ NaOH.

	Calculated for C ₂₅ H ₄₀ O ₇ ·COCH ₃ :	Found:
COCH ₃	9.76	9.27

⁷ *Zeitschr. f. physiol. Chem.*, xliii, p. 27, 1904-05.

Oxidation of cerebronic acid.

This was carried out according to the directions of Levene and Jacobs. The action takes about two hours on the steam bath; this is better than heating with a free flame, in that bumping and possible foaming are avoided. After the addition of sodium bisulfite, the mixture is acidified, the acids filtered off, dissolved in alcohol and changed into the sodium salts. The salts from 9 grams of cerebronic acid are extracted with a liter of boiling methyl alcohol. The residue is principally the salt of the unchanged cerebronic acid. The acid obtained from the filtrate is transformed into the lithium salt and this extracted with hot alcohol as long as a noticeable precipitate forms upon cooling the filtrate. Usually 1 liter is sufficient. The residue is the lithium salt of the new acid. This is converted into the free acid and purified through the lead salt as described above for cerebronic acid. It melts at 80°–81°. The possible identity of this acid with lignoceric acid has already been mentioned. This will be discussed in a future paper.

0.1196 gram substance gave 0.3428 gram CO_2 and 0.1416 gram H_2O .

0.5946 gram of the acid, dissolved in methyl alcohol and benzene, required 15.95 cc. of $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $\text{C}_{24}\text{H}_{48}\text{O}_2$:	Found:
C.....	78.20	78.15
H.....	13.20	13.15
M. W.....	368	373

Sodium salt. SOLUBILITIES. 100 cc. methyl alcohol contains, at its boiling point, 2.186 grams; at 0°, 0.243 gram.

100 cc. ethyl alcohol contains, at its boiling point, 1.870 grams; at 0°, 0.080 gram.

ANALYSIS. 0.1004 gram of the substance gave 0.2702 gram CO_2 and 0.1099 gram H_2O .

	Calculated for $\text{C}_{24}\text{H}_{48}\text{O}_2\text{Na}$:	Found:
C.....	73.77	73.40
H.....	12.13	12.25

Lithium salt. This salt begins to soften at 172° and melts at 173–178° (not sharp).

SOLUBILITIES. 100 cc. boiling ethyl alcohol contains 0.244 gram; at 0°, 0.06 gram. 100 cc. boiling methyl alcohol contains 0.210 grams and at 0°, 0.095 gram.

ANALYSIS. 0.1265 gram of the substance gave 0.3576 gram CO_2 and 0.1405 gram H_2O .

	Calculated for $\text{C}_{25}\text{H}_{50}\text{O}_2\text{Li}$:	Found:
C.....	76.92	77.10
H.....	12.65	12.40

Reduction of cerebronic acid.

Levene and Jacobs obtained a hydrocarbon which had nearly the required melting point for pentacosan. We have repeated the reduction under slightly different conditions and have obtained a hydrocarbon which shows the melting point given for pentacosan.

Lots of 5 grams of cerebronic acid, 25 cc. of hydriodic acid of specific gravity 1.96 and 2 grams of red phosphorus were heated in sealed tubes at 125° – 130° for eight hours. The solid contents of the tubes were filtered off, after diluting the acid with much water. In the first experiments the product was crystallized from alcohol, and then changed into the sodium salt by dissolving in absolute alcohol, neutralizing to phenolphthalein with sodium methylate and evaporating to dryness. The distillation of the sodium salt was accompanied with foaming, however, and so in the later experiments the product once crystallized out of alcohol was thoroughly dried in vacuum and distilled in the vacuum of a Geryke pump. The reaction was accompanied by decomposition and some foaming; the temperature was about 320° . The distillate was taken up in absolute alcohol, neutralized with sodium methylate, evaporated to dryness, and the dry mixture extracted with ether. The residue was changed into the free acid, again distilled and treated as above. The combined ether extract was dried over anhydrous sodium sulphate, the ether removed and the product distilled in vacuum. The distillate was recrystallized from ether or alcohol. It melted at 53 – 56° ; a second sample melted at 52 – 55° . Recrystallized from acetone it showed the same melting point. The product was then distilled, after the addition of a few cubic centimeters of sodium methylate solution and evaporation to dryness, the distillate recrystallized from acetone and thoroughly dried. It then melted at 53° – 54° . According to Marie* pentacosan should melt at 53.5° – 54° .

* *Bull. de la soc. chim.*, xv, p. 567, 1896.

0.1140 gram of the substance gave 0.3566 gram CO_2 and 0.1444 gram H_2O

	Calculated for $\text{C}_{24}\text{H}_{48}$:	Found:
C.....	85.13	85.30
H.....	14.89	14.18

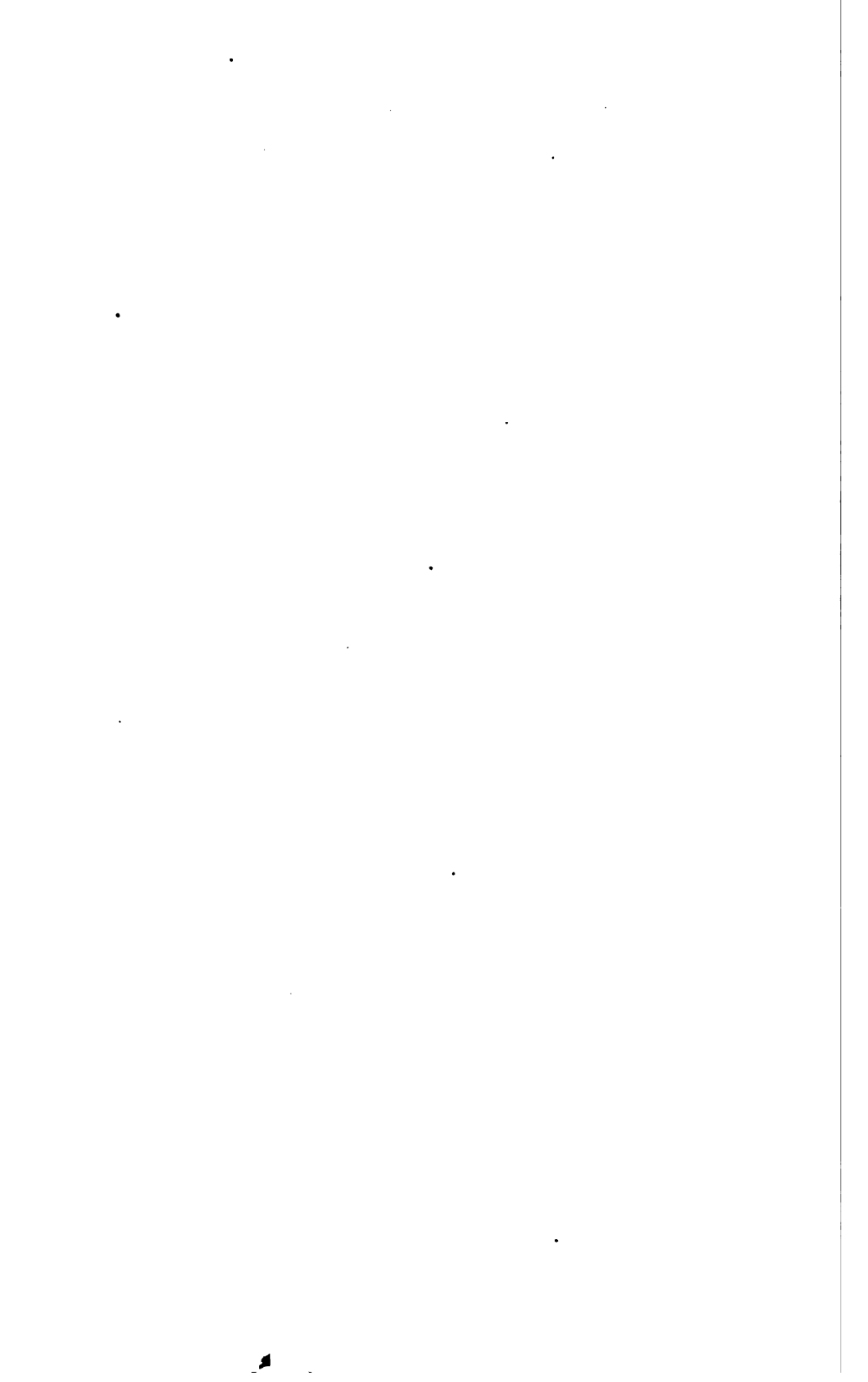
Reduction of $\text{C}_{24}\text{H}_{48}\text{O}_2$.

The above method was then applied to other fatty acids, in the hope that it might prove a general method for the reduction of acids to hydrocarbons. The result was disappointing.⁹ In the case of $\text{C}_{24}\text{H}_{48}\text{O}_2$, obtained from the oxidation of cerebronic acid, the product of the distillation was a mixture of a large amount of the ester of the acid with a small amount of the hydrocarbon. The ester was saponified by boiling with alcoholic potash, the solution evaporated to dryness and the mixture extracted with ether. A small quantity of hydrocarbon remained. This was recrystallized from alcohol and dried in the chloroform bath. It melted at 51° – 52° . According to Krafft¹⁰ $\text{C}_{24}\text{H}_{50}$ should melt at 51.1° .

	Calculated for $\text{C}_{24}\text{H}_{50}$:	Found:
C.....	85.10	85.27
H.....	14.90	14.79

⁹ The reaction was also tried with stearic and palmitic acids. The product in these cases was found to be a mixture of the unchanged acid with the ethyl ester. Thus it is probably the presence of the hydroxyl group which makes the reaction possible in cerebronic acid. It is also evident that the hydroxyl group is reduced simultaneously with or after the carboxyl group, otherwise the normal acid would be obtained.

¹⁰ *Ber. d. deutsch. chem. Gesellsch.*, xv, p. 1711, 1882.



CONTRIBUTIONS TO THE CHEMICAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM.

I. A COMPARISON OF THE BRAIN OF THE ALBINO RAT AT BIRTH WITH THAT OF THE FETAL PIG.

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(Received for publication, February 20, 1913.)

INTRODUCTION.

For the study of the progressive changes in the central nervous system during growth and senescence, the albino rat, on account of its small size, short span of life and its powers of rapid reproduction¹ is especially suited. Its growth processes are, moreover, strikingly like those of man, as has been brought out by the extensive investigations of Dr. Donaldson within the past few years. It was, therefore, decided to use this animal for a study of the chemical differentiation of the central nervous system during growth.

The youngest brains which could be conveniently collected for chemical analysis were those of rats just born. As it was not certain that the rat at this period of development was sufficiently immature (chemically undifferentiated) to serve as the starting point for such a growth series, it was suggested by my brother, Dr. Waldemar Koch, that the brain of the new born rat be compared chemically with the brain of the fetal pig, collected at various stages of fetal life.

By such a comparison we hoped to determine the physiological age of the rat at birth in terms of fetal pig material; and to obtain, possibly, from the pig fetus, material which would be more immature than the new born rat.

¹ H. H. Donaldson: President's Address, *Journ. of Nervous and Mental Disease*, xxxviii, p. 258, 1911.

MATERIAL AND METHODS.

The rat material was obtained from the Wistar Institute of Anatomy, which supplied the brains of rats of known age, from animals which had been raised under constant conditions; two factors which are absolutely essential for such a study. The material was collected by Dr. Hatai and the method used was that adopted by the Institute. This in brief is as follows: the rat was chloroformed; the skull opened from the dorsal side; the division between the brain and the cord made at the tip of calamus scriptorius; and the brain removed. The meninges of the brain were left intact. Such blood as it contained was, therefore, included in the weight. Immediately after removal the brain was placed in a closed weighing bottle, quickly weighed to within 10 mgms.² and transferred to a wide mouthed bottle of 300 cc. capacity containing absolute alcohol. The weighing bottle was weighed back and the difference recorded as the weight of the sample. As the weight of one brain from rats at this early age is 0.2 – 0.3 gram and as it takes at least from 25 to 50 grams to make one sample for analysis, a large number of brains had to be collected (100 brains of the rat at birth for one 25-gram sample). As this covered a period of several weeks it was necessary to heat the sample from time to time in a water bath kept at a temperature of 70°C. to insure a thorough penetration of the alcohol and sterilization. The amount of alcohol was so adjusted as to make the final concentration not less than 80–85 per cent. A well fitting cork stopper covered with tin-foil was now inserted and the bottle carefully shaken to insure a uniform mixture. The dates of collection of the samples were recorded, as the time a sample has been kept in some cases influences the analytical results.³ The tightly corked bottles were then shipped to the Laboratory of Biochemistry of the University of Chicago, where the samples were analyzed according to Koch's methods of tissue analysis.⁴

² A coarse weighing of the brain was permissible in this instance as it was not the exact brain weight that was sought but merely data for indicating roughly when the required amount of material had been obtained.

³ W. Koch: *Methods for the Quantitative Chemical Analysis of Animal Tissue*, *Journ. of the Amer. Chem. Soc.*, xxxi, p. 1340, 1909.

⁴ *Ibid.*, pp. 1329–64.

The fetal pig material was collected by my brother at the Chicago Stock Yards. The fetuses selected were 50, 100, and 200 mm. in length. The pregnant uterus was opened: and the fetuses removed. The neck-rump length of each litter was taken and if the average length corresponded to one of the three sizes mentioned above, the entire litter was taken, placed upon ice and in this chilled condition taken to the laboratory, where the brains were immediately removed, preserved and later analyzed according to the same methods used for the rat material.

RESULTS OF ANALYSES.

The results from the chemical analysis of the brains from the new born rat and the adult rat are given in table I: those of the 50, 100, and 200 mm. pig fetuses are recorded in table II, and table III gives the summary of all the averages which have been taken from the figures which were most consistent. The brain of the 200 mm. pig fetus was plainly more differentiated, it is therefore left out of table III and of the final discussion of results.

DISCUSSION OF CHEMICAL RESULTS.

Before taking up a comparison of the new born rat with the pig fetus it may be well to state, briefly, the chief chemical changes in nervous tissue during growth. It is well known that the chemical composition of a tissue varies with age and that the water content is one of the most important variables. Donaldson states that, "the progressive diminution of the percentage of water in the brain is a function of age and is not significantly modified by any conditions to which the animals have been thus far experimentally subjected."⁵ He suggested that this "is to be regarded as an index of fundamental chemical processes, which take place in the more stable constituents of the nerve cells."⁶ The principal chemical differences due to growth, noted by my brother, are, "a decrease in moisture, proteins, extractives, and ash as the brain increases with age, and an increase in cerebrosides, sulphatides,

⁵ H. H. Donaldson: On the percentage of Water in the Brain and in the Spinal Cord of the Albino Rat, *Journ. of Neurol. and Psychol.*, xx, p. 143, 1910.

⁶ *Ibid.*

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phosphatides, and cholesterol; in other words, an increase in substances which predominate in the fibres (medullated sheath) during growth."⁷ These same differences are to be found in all

TABLE I.

Relative proportion of the proximate constituents of the brain of the albino rat at birth and when adult.

	ALBINO RAT (AT BIRTH)		ALBINO RAT (ADULT)
Moist weight of one brain.....	0.25	0.25	1.667
Solids in per cent.....	10.42	10.42	21.9
Dry weight of one brain.....	0.026	0.026	0.380
Number of brains in sample.....	100	100	31

In relative proportions of solids.

Proteins.....	57.16	57.30	48.5
Phosphatides.....	14.8	15.6	22.0
Cerebrosides.....	0.0*	0.0	9.0
Sulphatides.....	1.5	1.4	4.6
Organic extracts }.....	16.5	19.3	9.8
Inorganic const. }.....			
Cholesterol }.....	(10.04)	(6.4)	(6.1)
Undetermined }.....			
Total S.....	0.96	1.04	0.58
Total P.....	1.82	1.92	1.39

Distribution of sulphur in per cent of total S.

Protein S.....	31.02	30.02	64.2
Lipoid S.....	3.2	2.8	15.6
Neutral S.....	49.14	47.26	14.2
Inorganic S.....	16.6	19.95	6.0

Distribution of phosphorus in per cent of total P.

Protein P.....	13.3		6.8
Lipoid P.....	33.2	33.0	67.6
Water-soluble P.....	53.5	53.6	25.6

* Mendel, L: *Amer. Journ. of Physiol.*, xxi, p. 104, 1908.

† By difference.

⁷ W. Koch and S. A. Mann: A Comparison of the Chemical Composition of Three Human Brains at Different Ages, *Journ. of Physiol.*, xxvi, pp. 1-3. (From the Proceedings of the Physiological Society, November 23, 1907).

TABLE II.

Relative proportions of the proximate constituents of the brain of the fetal pig at different ages.

Year of analysis.....	50 MM. PIG FETUS			100 MM. PIG FETUS			200 MM. PIG FETUS
	'11	'12	'12	'11	'12	'12	'11
Moist weight of one brain.....	0.40	0.43	0.47	1.8	1.91	2.15	10.1
Solids in per cent.....	8.75	9.87	9.04	9.1	8.98	8.98	
Dry weight of one brain	0.035	0.042	0.042	0.164	0.171	0.193	
Number of brains in sample.....	65	111	109	35	27	27	

In relative proportions of solids.

Proteins.....	56.6	58.2	54.61	51.5	51.81	52.34	43.8
Phosphatides.....	13.0	15.04	15.79	15.7	16.31	14.85	17.2
Cerebrosides.....							
Sulphatides.....	2.4?	0.8	1.05	1.8?	0.96	0.84	0.00?
Organic extract }	22.20	20.5	23.84	24.2	24.92	25.44	23.2
Inorganic const. }							
Cholesterol.....	2.4*	2.4*	2.4*	4.4*	4.4*	4.4*	
Undetermined†.....	(3.4)	(3.06)	(2.31)	(2.4)	(1.6)	(2.13)	(8.42)
Total S.....	0.67	0.59	0.58	0.59	0.57	0.55	0.55
Total P.....	1.74	1.85	1.90	1.76	1.91	1.82	1.45

Distribution of sulphur in per cent of total S.

Protein S.....	54.3	57.3	55.8	58.4	57.8	55.66	60.9
Lipoid S.....	7.2?	2.67	3.59	6.1?	3.36	2.98	0.00?
Neutral S.....	29.3	29.4	27.6	26.7	28.97	31.68	25.5
Inorganic S.....	9.0	10.67	13.0?	9.0	9.93	9.6	13.33

Distribution of phosphorus in per cent of total P.

Protein P.....		15.7	14.0		14.5	14.6	5.2
Lipoid P.....	31.6	32.4	29.6	35.5	34.5	31.6	46.3
Water-soluble.....	53.6	51.8	56.2		50.9	53.8	48.5

* Mendel, L.: *Amer. Journ. of Physiol.*, xxi, p. 103, 1908.

† By difference.

(?) Indicates doubtful result.

TABLE III.

Relative proportions of the proximate constituents of the brain of the fetal pig at different ages compared with the brain of the albino rat at birth. (Averages of the foregoing determinations.)

	PIG FETUS		ALBINO RAT	
	50 mm.	100 mm.	at birth	adult
Moist weight of one brain.....	0.433	1.90	0.25	1.667
Solids in per cent.....	9.22	8.99	10.42	21.9
Dry weight of one brain.....	0.039	0.171	0.026	0.380
Number of brains in sample.....	95	30	100	31

In relative proportions of solids.

Proteins.....	56.47	51.88	57.23	48.5
Phosphatides.....	15.41	15.62	15.2	22.0
Cerebrosides*.....	0.0	0.0	0.0	9.0
Sulphatides.....	0.92	0.90	1.45	4.6
Organic extract } Inorganic const. }	22.18	24.69	17.9	9.8
Cholesterol.....	2.4†	4.4†		
Undetermined†.....	(2.59)	20.49	(8.22)	(6.1)
Total S.....	0.585	0.57	1.00	0.58
Total P.....	1.83	1.83	1.87	1.39

Distribution of sulphur in per cent of total S.

Protein S.....	55.8	57.28	30.52	64.2
Lipoid S.....	3.13	3.17	3.0	15.6
Neutral S.....	28.7	29.11	48.2	14.2
Inorganic S.....	9.83	9.51	18.27	6.0

Distribution of phosphorus in per cent of total.

Protein P.....	14.8	14.55	13.3	6.8
Lipoid P.....	31.2	33.8	33.1	67.6
Water-soluble P.....	53.8	52.3	53.55	25.6

* Cerebrosides not determined in fetal brains. Not present according to Mendel.

† Mendel, L: *Amer. Journ. of Physiol.*, xxi, p. 103, 1906.

‡ By difference.

nervous tissue during growth and may therefore be used in making a comparison between the brain of the new born rat and that of the fetal pig to determine which is the more immature.

We may now proceed to consider in detail the comparison of the various constituents¹ in the brain of the new born rat and the pig fetus.

Water. The per cent of water in the brain of the new born rat is closely similar to, but a little lower than, that of either the 50 mm. or 100 mm. pig fetus. This would indicate that the rat is of about the same physiological age as these fetuses, since the differences are within the limits of error.

Protein. The per cent of protein in the total solids is higher in the brain of the new born rat than in either that of the 50 or the 100 mm. pig fetuses. Since the per cent of protein is highest in the youngest material, this is an indication that the rat's brain is less mature than that of the 100 mm. pig fetus, but not very different from the 50 mm. fetus.

Phosphatides. The per cent of phosphatides is the same in the new born rat as it is in the 50 and the 100 mm. pig fetus. This would indicate a close agreement in physiological age between these two. This is the lowest phosphatide content yet obtained in an analysis of the brain tissue and approaches that observed in the suprarenal, which, among all the organs, comes closest to that of nervous tissue in chemical composition.

Cerebrosides. These are absent in both the new born rat, and in the pig fetus, as is to be expected in nervous tissue before myelination.

Sulphatides. The percentage of sulphatides is about the same in the new born rat as in the pig fetus, which indicates the same age.

Organic extractives and inorganic constituents. These are somewhat higher in the pig fetus than in the new born rat and, except as this is associated with the greater per cent of lymph in the embryonic material, it would indicate it to be more immature than the new born rat.

¹ The nature and significance of the constituents will be discussed in the third paper of this series.

Cholesterol. The figures for cholesterol were not determined by me, but were taken from Mendel⁹ and incorporated here for the sake of completeness.

This leaves *undetermined* from 2 to 3 per cent which is not more than would be expected in the errors involved in making so many determinations from one tissue and calculating approximate constituents from assumed factors.

The distribution of sulphur in per cent of total sulphur is widely different in the two forms, but as this is not correlated with age but is apparently a species peculiarity, the results are not out of harmony with the foregoing conclusions.

The distribution of phosphorus between the protein, lipoid and water-soluble phosphorus is closely similar in the rat and the 50 and 100 mm. pig fetus, showing the physiological ages to correspond.

The remarkably high figure for neutral and inorganic sulphur in the rat at birth requires an explanation but it is not possible to give this with the data so far at hand.

The general conclusions from these figures are, that from a chemical standpoint the brain of the new born rat is about as immature as that of the 100 mm. pig fetus, being on the whole a little less differentiated than the latter.

The differences between the brain of the 50 mm. and the 100 mm. pig fetus are not marked, and this would indicate that there occurs between these ages an increase in weight unaccompanied by any significant change in chemical composition. This would correspond with the results of Mendel¹⁰ and Raske¹¹ who found that in the brains from these young fetuses there is no chemical distinction between grey and white matter. Moreover the brain of the 50 mm. pig fetus is the youngest which it is practicable to obtain for analysis and even at this age the tissues are so watery and filled with lymph that some error is thereby introduced in the analysis of the constituent tissues.

Since the brain of the 50 mm. pig fetus shows no material differences from that of the 100 mm. pig fetus and the latter is no more

⁹ L. B. Mendel and Charles S. Leavenworth: Chemical Studies on Growth. IX. Notes on the Composition of Embryonic Muscular and Nervous Tissues. *Amer. Journ. of Physiol.*, xxi, p. 103, 1908.

¹⁰ *Ibid.*

¹¹ Raske: *Zeitschr. f. physiol. Chem.*, x, p. 340, 1886.

immature chemically than that of the new born rat, it appears that the new born rat's brain is as young nervous material as can conveniently be analyzed at present: and it forms, therefore, a convenient starting point for the study of the chemical differentiation of the central nervous system during growth.

CHEMICAL RESULTS CONFIRMED BY PHYSIOLOGICAL AND ANATOMICAL DATA.

It is astonishing that chemically the brain of the new born rat should be as immature as that of the 100 mm. pig fetus, but, surprising as this fact is, it is substantiated by a comparison of the structure of the cerebellum of these two animals and of their behavior at the time of birth.

It is a well-known fact that the rat is born in a very immature state, with its eyes shut, and when first born, is capable only of movements involved in sucking, bending the body and tail and making a squeaking noise.¹² The pig, on the other hand, "is born with its eyes open and requires no assistance as a rule in making its start in life. It is more or less able to walk around as soon as born."¹³ Such a state of activity in the rat is not reached until the period of weaning 17-21 days after birth.

This difference in physiological behavior is correlated with the relative development of the cerebellum of the two animals; particularly as indicated by the development and transformation of the outer granular layer of cells. A comparison of this layer in both animals, founded on the observations of Addison¹⁴ who studied the different layers of the cerebellum in the albino rat, and of Takasu¹⁵ who studied these same layers in the pig fetus, brought out the following facts:

¹² Wm. H. F. Addison: The Development of the Purkinje Cells and the Cortical Layers in the Cerebellum of the Albino Rat, *Journ. of Comp. Neurol.*, xxi, p. 476, 1911.

¹³ Forbes: personal communication.

¹⁴ Wm. H. F. Addison: *Journ. of Comp. Neurol.*, xxi, p. 464, 1911.

¹⁵ K. Takasu: Zur Entwicklung der Ganglienzellen der Kleinhirnrinde des Schweines, *Anat. Anz.*, xxvi, pp. 225-32, 1905.

	RAT	PIG
First appearance of cells in outer granular layer.....	19-day fetus	50 mm. fetus
Division of layer into two zones inner and outer.....	At birth	100 mm. fetus
Disappearance of cells from inner zone of layer.....	21 days after birth	300 mm. fetus

The first appearance of the cells in the outer granular layer of the cerebellum in the rat is in the 19-day fetus, and in the pig in the 50 mm. pig fetus. At this time the cells are settled in a thin layer (two rows deep) around the outer edge of the cerebellar cortex. This layer increases until a considerable depth is filled in with cells which soon separate into two strata, an outer and an inner; this separation takes place in the rat at birth, and in the pig fetus when it is 100 mm. in length. The cells from the outer granular layer now begin to migrate to the inner granular layer and the disappearance of the cells from this outer granular layer, which corresponds with the time of securing motor control in an animal, occurs in the rat at the twenty-first day of life, and in the pig when this is from 200 to 300 mm. in length, or at birth. These facts show, therefore, that the new born rat is as developed with respect to motor activity as the 100 mm. pig fetus, and that the rat at weaning (17-21 days after birth) and the pig at birth are at corresponding physiological ages. The conclusion from this anatomical comparison, namely, that the 100 mm. pig fetus and the rat at birth are of like physiological age, fully confirms, therefore, the conclusion drawn from both chemical and physiological evidence already adduced.

We now ask the question, how far this result, that the nervous system of the new-born rat is chemically as old as that of the 100 mm. pig fetus, agrees with observations made by Donaldson, that the rate of growth and percentage of water in the mammalian nervous system (represented by the brains of man and the rat) agree in the two forms at equivalent ages, thus indicating that the nervous systems are in corresponding physiological states at equal fractions of the life cycles.¹⁶

¹⁶ H. H. Donaldson: A Comparison of the White Rat with Man in respect to the Growth of the Entire Body, *Boas Anniversary Volume*, 1906, pp. 5-26.

It remains therefore to inquire whether the chemical and behavior relations between the rat and pig which have just been pointed out, occur at equivalent ages in these two forms.

Great difficulty was experienced in finding any statements concerning the age of the pig fetuses. The statements of different authors did not always agree, but the two which agreed closest were those of Bradley¹⁷ and Coe.¹⁸ Bradley compared the length of the embryos with the time from coition; Coe estimated the age from the rate of development of embryos of other mammals. While considerable uncertainty thus attaches itself to these figures¹⁹ it may be assumed that the 50 mm. pig fetus is about 40 days old from conception: the 100 mm. fetus is 55–62 days; and the 200 mm. fetus is from 88–90 days from conception.

We find in the rat the period of gestation is 21 days and its span of life three years (Donaldson), or a total age of 1116 days; in the pig the period of gestation is 125 days and its normal span of life, as far as could be ascertained, is 20 years,²⁰ or 7425 days. The rat, therefore, lives about one-sixth as long as the pig. Assuming that the rat at birth has lived $\frac{21}{1116}$, or $\frac{1}{53}$, of its total life, the 60-day pig fetus will have lived $\frac{60}{7425}$, or $\frac{1}{123.75}$, of its life. It appears then, if the total length of life given is correct for both animals and the numbers used for the divisors in each case are really comparable as they stand, that we do not have corresponding physiological conditions of the brain at equivalent ages, for these brains

¹⁷ O. C. Bradley: On the Development of the Hind Brain of the Pig, *Journ. of Anat. and Physiol.*, xl, Part I, p. 1.

¹⁸ Mendel refers to Professor Coe in Chemical Studies on Growth. I. The Inverting Enzymes of the Alimentary Tract, especially in the Embryo, *Amer. Journ. of Physiol.*, xx, p. 90, 1907–1908.

¹⁹ Bradley makes the statement, that "although the age of the different embryos is given, it is not intended that it should signify more than the time which elapsed between the time of coition and the time when the mother was destroyed. . . . In embryos taken from two litters it not infrequently happens that those which should be further advanced in development, judging from the period which has elapsed since sexual congress took place, are as backward, or even more backward, than those of the 'younger' litter." A more definite way to determine the age of an embryo would be, according to Mall, by ossification. No data were available however for a comparison between the rat and the pig.

²⁰ Longevity, *Encyclopædia Britannica* (Eleventh Edition), xvi, p. 975.

are found to be in corresponding states at the $\frac{1}{15}$ and the $\frac{1}{15}$ part of the total life cycles. Had the relation, in the form stated above, held, these fractions should have been identical. It is only fair to add, however, that in view of the absence of precise information concerning the pig and in view of the fact that the early days of gestation are used for cell division accompanied by only slight differentiation, too much stress should not be laid on the relation here given.

On the other hand, instead of taking the end of life as the fixed point of our calculations, we may consider the time when motor control is obtained to indicate closely corresponding states of the central nervous system. In the rat, motor control is obtained at 42 days from conception, and in the pig at 125 days, that is, at birth. If the law of corresponding states is correct, the nervous system of these two animals should be in corresponding conditions at the same fractions, either $\frac{1}{3}$, $\frac{1}{2}$, or $\frac{2}{3}$ of these periods. This is found to be the case, for the rat is born after 21 days' gestation. This would be just half way between the two fixed points of conception and time of gaining motor control and this corresponds in the pig to just one-half of its gestation period or about 62 days, which is the age of the 100 mm. pig.

It was actually found, both chemically and anatomically, that the nervous systems of these two animals were in the same state of development at these respective periods and it appears from these observations that Donaldson's law may hold, if put in the form: the nervous systems of mammals are in the same physiological state at equal fractions of their total periods of development.

In conclusion it gives me great pleasure to thank Dr. H. H. Donaldson and Prof. A. P. Mathews for their many suggestions in connection with this problem and for their aid in getting this paper ready for publication. The problem itself was suggested to me by my brother and forms the first of a series of papers which are to follow from time to time, on the chemical differentiation of the central nervous system, and on which he was engaged at the time of his death. The work was carried out in the Laboratory of Biochemistry and Pharmacology of the University of Chicago and was aided by a grant from the Wistar Institute of Anatomy and Biology, Philadelphia.

SUMMARY.

1. A quantitative determination of the constituents of the brain of the albino rat at birth shows it to be chemically as undifferentiated as the brain of a 50 mm. or 100 mm. pig fetus.

2. There is little difference in chemical composition between the 50 mm. and the 100 mm. pig fetus brain.

3. Since the 50 mm. fetus brain is the youngest which can be analyzed and this closely resembles the 100 mm. fetus, and this in turn is no more immature than the new born rat, it appears that the brain of the new born rat is sufficiently immature to serve as a starting point in a study of the chemical differentiation of the brain during growth.

4. That the brain of the new born rat is as immature as the 100 mm. pig embryo is shown, also, by the similarity of the changes in the outer layer of cells of the cerebellar cortex in both animals previous to gaining motor control, and by the animal's behavior at this period.

5. If the nervous systems are assumed to be in corresponding states when motor control is obtained, and Donaldson's law is correct, that the nervous system is in the same state at corresponding physiological ages, then the brain of the rat at birth should correspond chemically with the 100 mm. pig fetus brain. This is found to be the case.

CONTRIBUTIONS TO THE CHEMICAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM.

II. A COMPARISON OF TWO METHODS OF PRESERVING NERVE TISSUE FOR SUBSEQUENT CHEMICAL EXAMINATION.

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(Received for publication, February 20, 1913.)

With valuable biological material it is sometimes desirable to make water estimations and the estimations of the other constituents on the same sample. This can be done somewhat indirectly if the method already described¹ of placing the fresh, weighed tissues immediately in 95 per cent alcohol is used.

To see whether tissues in which the water had been determined by drying could thereafter be analyzed by the methods referred to above and would yield the same proportion of the various constituents as these same tissues treated by the alcohol method, an analysis was made of the brains and spinal cords of albino rats which had been dehydrated in these two ways. The possible drawbacks of the heat method of determining moisture, namely, the oxidation or decomposition of part of the material and the evaporation of volatile constituents, are obvious, but we had no definite knowledge of how serious the errors involved in the method might be in practice.

To determine to what extent these changes took place and what they were, we analyzed material which had been dried at 95°C. for one week and which at the end of this time had been placed in alcohol, and compared it with similar material which had been placed directly in alcohol. The results are given in the table.

It may be seen by a comparison of the results of the two analyses in the table, that decompositions seriously affecting the analyses are produced by heat drying, particularly in the case of the brain. The differences are most marked in the phosphorus compounds.

¹ Koch, W.: *Journ. Amer. Chem. Soc.*, xxxi, pp. 1353-4, 1909.

Comparison of brains and cords dried at 95°C. with brains and cords placed directly in alcohol without heating.

	ENCEPHALON		CORDS	
	Direct into alcohol	Dried at 95°C.	Direct into alcohol	Dried at 95°C.
Laboratory number.....	W 13	W 18	W 9	W 20

In per cent of total solids.

Proteins.....	48.5	47.9	32.8	29.6
Phosphatides.....	22.0	16.2	25.3	22.1
Cerebrosides.....	8.4	(?)	12.5	14.4
Sulphatides.....	4.5	4.6	7.0	6.7
Organic ext. } Inorganic const. }	9.8	12.4	7.6	8.0
Undetermined lipoids.....	6.8*		14.8*	19.2*
Total S.....	0.58	0.59	0.45	0.42
Total P.....	1.39	1.31	1.44	1.42

Distribution of sulphur in per cent of total S.

Protein S.....	63.8	64.6	53.7	53.3
Lipoid S.....	15.6	15.6	30.9	32.1
Neutral S.....	14.5	14.0	10.3	9.5
Inorganic S.....	6.1	6.0	5.0	5.0

Distribution of phosphorus in per cent of total P.

Protein P.....	6.8	8.1	5.6	5.0
Lipoid P.....	67.6	55.1	77.4	69.2
Water Sol. P.....	25.6	36.8	17.0	25.8

* Obtained by difference.

By drying there has been a destruction of the phosphatides involving a change in the distribution of phosphorus in per cent of total phosphorus; that is, a considerable amount of lipid phosphorus is changed to water-soluble phosphorus. There was no change in the sulphur distribution. It will be noticed that the phosphatides of the cord are more resistant to heat than those of the brain, a point of sufficient interest to justify repetition.

We conclude, then, that the determination of water by drying at 95°C. cannot safely be used, if it is desired to determine in the same sample the relative proportions of the solid constituents; and that the indirect method already described is far superior for this purpose.

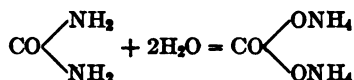
A RAPID CLINICAL METHOD FOR THE ESTIMATION OF UREA IN URINE.

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(Received for publication, February 20, 1913.)

As is well known, the conversion of urea into ammonium carbonate can be brought about by an enzyme found in numerous bacteria,¹ fungi² and higher plants.³



An enzyme solution exerting this activity is easily and conveniently prepared from the soy bean (*Glycine hispida*).⁴ By extracting the ground tissue with acidified water and filtering, a perfectly clear solution is obtained which under proper precautions can be preserved for a considerable length of time. A solution in which urea is to be determined is treated with a portion of this extract and, after the conversion into ammonium carbonate is complete, the alkalinity of the solution is determined by titration with standard acid using an indicator which is not appreciably sensitive to

¹ Musculus: *Compt. rend. de l'Acad. des Sci.*, lxxxii, p. 334, 1876; Leube: *Virchow's Archiv*, c, p. 540, 1885; Lea: *Journ. of Physiol.*, vi, p. 136, 1886; Miquel: *Bull. de la soc. chim. de Paris*, xxix, p. 387; xxxi, p. 391; xxxii, p. 136; also, *Compt. rend. de l'Acad. des Sci.*, cxi, pp. 397, 501.

² Shibata: *Beitr. z. chem. Physiol. u. Path.*, v, p. 384, 1904.

³ Takeuchi: *Journal of the College of Agriculture, Tokyo*, i, p. 1, 1909; Keisel: *Zeitschr. f. physiol. Chem.*, lxxv, p. 169, 1911; Zemplén: *Zeitschr. f. physiol. Chem.*, lxxix, p. 229, 1912; *Zeitschr. f. angew. Chem.*, xxv, p. 1560, 1912.

⁴ This enzyme was discovered in the seeds and seedlings of the soy beans by Takeuchi who employed its hydrolyzing power for the manufacture of ammonium sulphate from urine: *loc. cit.* and also *Chem. Zeitung*, xxxv, p. 406, 1911.

carbonic acid. From the amount of standard acid used, the amount of urea originally present can be ascertained by an obvious calculation.

With solutions of pure urea the figures obtained are practically theoretical and, even when applied to urine itself, the results are all that could be desired in a clinical method, the divergence from the theoretical being about 2 per cent.

The procedure to be described is peculiarly useful in its application to pathological urines since the presence of glucose and protein, usually so annoying in the estimation of urea, are without influence here and need not even be removed.

A large number of methods based upon different principles have been devised for the estimation of urea in urine. For scientific investigations, the one proposed by Folin⁵ in its various modifications, the Mörner-Sjöqvist⁶ method and its combination with Folin's for urines containing sugar, and finally the procedures devised by Benedict⁷ have been extensively used. All of these methods, however, necessitate an independent determination of the preformed ammonia in the urine, and in general are not sufficiently simple to find favor for rapid clinical work. The various methods, based upon the decomposition of urea by an alkaline solution of sodium hypobromite and a measurement of the nitrogen evolved, are, therefore, still used for clinical purposes. As is well known, however, they are very inaccurate and the results are vitiated by the presence of ammonium salts.

Miquel⁸ in his investigations on the various urea-fermenting bacteria suggests that filtered cultures of *Micrococcus ureae* could be utilized to convert the urea of the urine into ammonium carbonate and to estimate its amount. However, he cites but one experiment in this connection. Hudson⁹ has proposed a very accurate method for the determination of cane sugar by means of

⁵ Folin: *Zeitschr. f. physiol. Chem.*, xxxii, p. 504, 1901; xxxvi, p. 333, 1902; xxxvii, p. 548, 1902-03; *Amer. Journ. of Physiol.*, xiii, p. 45; also, this *Journal*, xi, p. 45, 1912.

⁶ Mörner: *Skand. Arch. f. Physiol.*, xiv, p. 297, 1903.

⁷ Benedict and Gephart: *Journ. of Amer. Chem. Soc.*, xxx, p. 1760, 1908; Benedict: this *Journal*, viii, p. 405, 1910-11.

⁸ *Compt. rend. de l'Acad. des Sci.*, cxi, p. 501, 1890.

⁹ *Journ. of Ind. and Eng. Chem.*, ii, p. 143, 1910.

the invertase of yeast. The enzyme solution employed hydrolyzes cane sugar completely, while it is without action upon maltose, lactose, starch, dextrins, pentosans, and the natural glucosides. The use of the fermentation of glucose brought about by yeast has been employed clinically for the determination of sugar in pathological urines.

EXPERIMENTAL.

Preparation of the enzyme solution. The soy beans are ground to a fine powder which can be preserved in well-stoppered dry bottles for months without appreciable loss of activity. Twenty-five grams of this powder are mixed with 250 cc. of distilled water, and allowed to stand with occasional agitation for about an hour. Twenty-five cc. of $\frac{N}{10}$ hydrochloric acid are now added, and the mixture allowed to remain a few minutes longer (best in a water bath at about 35°), when a large proportion of the protein of the bean extract is precipitated. The mixture is filtered; the filtrate treated with a few drops of toluene and preserved for use in a stoppered vessel. On standing the originally clear fluid becomes opalescent, and finally a precipitate is formed, but the solution remains sufficiently active for use in the method at least five days after its preparation when kept at the room temperature.¹⁰ This solution is alkaline to methyl orange, and 2 cc. generally require from 0.28 to 0.34 cc. of $\frac{N}{10}$ hydrochloric acid for neutralization. This factor should be determined once for 2 cc. of each preparation and can then be employed as a correction as long as the solution is used. The alkalinity is apparently constant from day to day. If for any reason the extract should not be distinctly alkaline to methyl orange, less acid should be used in its preparation, as an extract which reacts acid to methyl orange is scarcely active.

Execution of the method. Two 5 cc. portions of the urine are measured into flasks of 200–300 cc. capacity and diluted with distilled water to about 100–125 cc. Two cc. of enzyme solution are added to one flask, a few drops of toluene to each and the solution allowed to remain, well stoppered, at room temperature over-

¹⁰ The enzyme solution loses its activity much more rapidly at a higher temperature (35°), and would, therefore, probably keep better in an ice chest. I am at present investigating the problem of preparing a purer solution and finding means of preserving its activity.

night. The fluid in each flask is titrated to a distinct pink color with $\frac{N}{10}$ hydrochloric acid, using methyl orange as an indicator. The amount of hydrochloric acid required for the contents of the flask containing the urine and enzyme solution less the amount used for 5 cc. of urine alone and that previously determined for 2 cc. of enzyme solution, corresponds to the urea originally present in the sample of urine. Since 1 cc. of $\frac{N}{10}$ hydrochloric acid is equivalent to 3 mgm. of urea, the number of cubic centimeters required multiplied by 0.6 gives the value of urea expressed in grams per liter of urine. The completeness of the conversion of the urea into ammonium carbonate can be tested at any time by performing one determination as described and a duplicate which is allowed to stand at 35°–38°. The results of the two experiments should be identical provided the hydrolysis is complete, but if the enzyme has not converted all the urea in the first determination, the second will give higher results. The same purpose is attained, of course, by allowing the second determination to proceed at the same temperature but some hours longer than the first.

Length of time necessary for the complete conversion. The time required for complete hydrolysis of the urea depends upon the quantity of urine used, the concentration of the urea, the amount of enzyme present, and the temperature of action. The velocity of the reaction is approximately twice as rapid at 35° as at 25°, and directly proportional to the enzyme concentration within certain limits. As shown by the following data, 5 cc. of urine or 5 cc. of a 2 per cent urea solution required about 3 hours for complete hydrolysis at a temperature of 35°.

TIME	2 PER CENT UREA SOLUTION: CORRECTED VALUE: CC. 0.1 N HCL	URINE: CORRECTED VALUE CC. 0.1 N HCL
hours		
1	23.45	26.03
2	32.55	37.00
3	33.15	40.74
4		40.78
24	33.10	

The conversion is complete in less than 1 hour at 35°, when 10 cc. of the enzyme solution are employed instead of 2 cc.

TIME	CORRECTED VALUE: CC. 0.1 N HCL
<i>minutes</i>	
45	33.26
80	32.68

A cloudiness, however, is produced on titrating a solution containing 10 cc. of the enzyme mixture, which renders the end point uncertain and the procedure less accurate. With the use of only 2 cc. of enzyme solution this cloudiness is scarcely noticeable. If more rapidity is required than is attained by the method as outlined, digestion for three hours at a temperature of 35°–38° will suffice, or, if accuracy is to be sacrificed to rapidity, less urine and more enzyme solution can be employed.

Application of the method to pure urea solutions. The following table explains itself.

GRAMS UREA 100 CC. USED	CC. 0.1 N HCL REQUIRED	CORRECTION FOR ENZYME SOLUTION	GRAMS UREA PER 100 CC. FOUND
0.500	8.55	0.28	0.4962
1.000	16.79	0.28	0.9906
2.000	33.50	0.30	1.9920
2.000	33.45	0.30	1.9890

Application of the method to urine. The following results obtained with a sample of normal urine are a fair example of the agreement by this method. Two duplicate determinations carried out at room temperature gave 2.190 and 2.187 grams of urea per 100 cc. Two similar determinations on the same sample but carried out at 37° gave 2.200 and 2.173 grams per 100 cc. The effect of adding a known amount of urea to the urine is shown below. Two cc. portions of urine were used and 5 or 10 cc. portions of a 1 per cent urea solution.

AMOUNT UREA ADDED EXPRESSED IN CC. 0.1 N HCL	REQUIRED BY URINE ALONE: CC. 0.1 N HCL	REQUIRED BY URINE AND UREA CC. 0.1 N HCL	DIFFERENCE UREA FOUND EXPRESSED IN CC. 0.1 N HCL
16.67	15.35	31.86	16.51
16.67	16.52	33.00	16.48
33.33	15.35	48.55	33.20

The method was applied to solutions of composition stated below. These solutions contained approximately the amount of urea normally present in the urine.

	SOLUTION I GRAMS PER LITER	SOLUTION II GRAMS PER LITER	SOLUTION III GRAMS PER LITER	SOLUTION IV GRAMS PER LITER
Urea.....	20.00	10.00	20.00	20.00
Creatinine.....	0.72	0.36		
Hippuric acid.....	0.50	0.25	0.50	
Uric acid.....			0.50	
NaCl.....	10.00	5.00	10.00	10.00
KH_2PO_4	1.40	0.70	1.40	1.40
Na_2HPO_4	1.40	0.70	1.40	1.40
K_2SO_4			1.50	1.50
MgSO_4			0.25	0.25
NH_3			0.50	

Solution I contained also potassium sulphate, potassium acetate and acetic acid, as the creatinine solution was prepared from creatinine zinc alum by precipitating the zinc with hydrogen sulphide in the presence of potassium acetate.

Solutions I, II and IV were acid to litmus and alkaline to methy orange, while solution III was alkaline to both litmus and methy orange, the former being the condition of acidity in normal urine

SOLUTION NO.	CORRECTED VALUE CC. 0.1 N HCl FOUND	THEORETICAL VALUE CC. 0.1 N HCl CALCULATED
I.....	32.85	33.33
I.....	32.75	33.33
I.....	32.89	33.33
II.....	16.42	16.67
II.....	16.45	16.67
II.....	16.31	16.67
III.....	32.98	33.33
III.....	32.86	33.33
III.....	32.86	33.33
III.....	32.81	33.33
IV.....	32.94	33.33
IV.....	32.97	33.33
IV.....	32.89	33.33
IV (boiled).....	33.26	33.33
IV (boiled).....	33.21	33.33

The uric acid and hippuric acid were converted into the sodium salts by the addition of the theoretical quantity of $\frac{N}{10}$ sodium hydroxide.

The last two samples were treated with an excess of $\frac{N}{10}$ hydrochloric acid, boiled to expel the carbon dioxide, cooled, and titrated with alkali.

Application of the method to urine containing sugar and albumin. Variable quantities of egg albumin and glucose were added to a sample of normal urine, and the urea determined.

ADDED TO 5 CC. URINE	5 CC. URINE ALONE REQUIRED CC. 0.1 N HCL	5 CC. URINE AFTER HYDROLYSIS RE- QUIRED CC. 0.1 N HCL	CORRECTED VALUE CC. 0.1 N HCL
	1.85	40.66	38.51
	1.91	40.80	38.59
0.2 cc. egg-albumin.....	2.12	40.85	38.43
1.0 cc. egg-albumin.....	2.80	41.48	38.38
0.1 gram glucose.....	1.90	40.65	38.45
0.1 gram glucose.....	1.90	40.77	38.57
0.5 gram glucose.....	1.87	40.74	38.57
0.5 gram glucose.....	1.87	40.81	38.64

Effect of the carbon dioxide on the titrations. The results obtained on pure urea solutions, urea solutions added to urine, and urea in the presence of various nitrogenous substances and salts are consistently 1 to 2 per cent lower than the theoretical values. In the two experiments in which the carbon dioxide was removed by boiling, the figures differ from the theoretical by only a few tenths of one per cent. The lower values are undoubtedly caused by the effect of the carbonic acid on the indicator in the presence of neutral salts, especially the ammonium chloride formed during the titration.¹¹ The magnitude of this error was determined by titrating a solution of ammonium carbonate with and without the removal of the carbon dioxide.

¹¹ This is, of course a well-known fact. However, the extent of the error involved by using methyl orange with carbon dioxide in the presence of neutral salts is generally underestimated. Acree and Brunell have already pointed out this fact (see *Amer. Chem. Journ.*, xxxvi, p. 120, 1906). The fact that the reaction may be slightly reversible must not be overlooked in this connection, although no evidence of such reversibility has so far been obtained.

Ten cc. of ammonium carbonate solution required 29.78 and 29.81 cc. of $\frac{N}{10}$ HCl. Removing the carbon dioxide, 10 cc. required 30.03 and 30.05 cc. of $\frac{N}{10}$ HCl. The error is, therefore, about 0.8 per cent. Removing the carbon dioxide would, of course, increase the accuracy of the method, but as the control with 5 cc. of urine alone cannot be subjected to the same treatment (adding excess acid, boiling, and retitrating) on account of an hydrolysis of the urea, it is not introduced as an essential feature. The carbon dioxide could also be removed by passing a rapid current of air through the acidified solution, or pumping out *in vacuo*.

The use of this refinement, however, is scarcely desirable when dealing with urine since results within an error of 2 per cent¹² can be secured by the more rapid procedure.

¹² The method described in this paper is recommended for the rapidity with which it can be executed and the very fair degree of accuracy secured. But by the introduction of certain refinements (the distillation of ammonia before the titration is made) the method can be made sufficiently accurate for scientific work such as the determination of urea in the blood. I am at present engaged with problems of this nature.

THE LIPINS OF THE HEART MUSCLE OF THE OX.¹

By JACOB ROSENBLOOM.

(*From the Laboratory of Biochemistry of the University of Pittsburgh, Pittsburgh, Pa.*)²

(Received for publication, February 21, 1913.)

The question of how lipins are combined in cellular material is of great importance. The writer in another connection³ has reviewed our knowledge of all the known combinations in which lipins may exist in cellular material.

On account of the labile nature of these substances it is possible that our methods of extraction may change the true nature of these substances. Recently MacLean and Williams,⁴ by extracting dog's liver in the cold with ether and alcohol, show that as much as 84 per cent of the total extract is of the nature of phosphatide (phospholipin) and conclude "that the essential fat of the liver, and probably of certain other organs is really phosphatide, and under certain circumstances, if care be taken to avoid disintegration during the process of extraction, it may be practically the only one found in any appreciable amount in the combined part of the fat."

In light of the recent observations of Leathes⁵ and his co-workers, which show the liver to be an active agent in the elaboration of

¹ A preliminary account of this work was published in *Science*, xxxiv, p. 221, 1911; *Biochem. Bull.*, i, p. 114, 1911.

² Most of the analytical data presented in this paper were obtained while working in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York City.

³ *Studies in Cancer and Allied Subjects, Conducted under the Auspices of the George Crocker Special Research Fund*, Volume III, Department of Biological Chemistry.

⁴ *Biochem. Journ.*, iv, p. 455, 1909.

⁵ Mottram: *Journ. of Physiol.*, xxxviii, p. 281, 1909; Leathes: *Arch. f. exp. Path. u. Pharm.* (*Schmiedeberg's Festschrift*), 1908, p. 327; *Lancet*, clxxvi, p. 594, 1909; Hartley: *The Fats*, 1910; *Journ. of Physiol.*, xxxvi, p. 17, 1907; *ibid.*, xxxviii, p. 353, 1909; Hartley and Mavrogordato: *Journ. of Path. and*

the complex "fats" and that it effects the desaturation of fatty acids, it is possible that this organ might contain a larger amount of phospholipin than other organs supposedly not so important in the elaboration of substances of the nature of the complex fats; and that it was not so much a question of the disintegration of the phospholipins during extraction as it was whether the liver "fat" might be different from the fat of other organs.

Paton⁶ however obtained the following results as regards the amount of lecithin in the ether extract of liver of rabbits. A rabbit liver was divided into two parts, one part being dried at 38°C. for forty-eight hours, the other dried for eight hours on a water bath. The liver dried at 38° yielded 56.1 per cent of lecithin in the ether extract and the liver dried on the water bath gave 58.4 per cent of lecithin in the ether extract, showing that very little change had taken place.

The following experiments show that *only about 40 per cent of the ether and alcohol extract of heart muscle of the ox is composed of phospholipin and practically no difference in this percentage was obtained on comparing the extractions carried out in the cold with those carried out at the boiling point of the solvent.*

The preparation of the organs for analysis, the extractions and the estimation of the phospholipins were carried out according to the methods described in a former paper.⁷ The extractions in the cold were conducted as follows: the material was placed in a thick-walled liter bottle, about 500 cc. of solvent added, and the bottle shaken vigorously for four periods, of fifteen minutes each, during every twenty-four hours. The extract was then decanted and a fresh supply of the solvent added. This was continued until a fresh supply of solvent yielded no residue when carried to dryness.

I thought it would be of interest also to carry out extractions, first with ether, then with alcohol followed by ether, and compare this extract with that obtained by first extracting with alcohol, then with ether followed by alcohol.

Bact., xii, p.371, 1908; Kennaway and Leathes; *Proc. Roy. Soc. Med.*, February, 1909; Leathes and Wedell: *Journ of Physiol.*, xxxviii, 1909, Proceedings. p. xxxviii.

⁶ *Journ. of Physiol.*, xix, p. 181, 1896.

⁷ Hanes and Rosenbloom: *Journ. of Exp. Med.*, xiii, p. 355, 1911.

The following tables contain the results obtained in this study.

TABLE 1.

A. Extraction with ether followed by alcohol and then ether, at room temperature.

NO.	WEIGHT OF DRIED HEART MUSCLE	ETHER EXTRACT	ALCOHOL AND ETHER EXTRACT	TOTAL EXTRACT	PERCENTAGE OF LIPINS IN TISSUE	PERCENTAGE OF PHOSPHOLIPINS IN THE LIPIN EXTRACT
	grams	grams	grams	grams		
1	56.4450	7.4140	2.4505	9.8645	17.48	40.54
2	47.1715	6.1230	1.8975	8.0205	17.00	40.80

B. Extraction with ether followed by alcohol and then ether in Soxhlet apparatus, at boiling point of solvent.

1	61.5260	9.0020	3.9050	12.9070	20.45	41.56
2	61.3020	8.9005	3.6380	12.5385	20.98	41.84

On comparing the above data it is evident that, by extracting the tissue at the boiling point of the solvent, about 3 per cent more lipins are obtained than when the extraction is carried out at the room temperature. This may be due to the breaking up of certain protein-lipin compounds at this temperature. However, the percentage of phospholipins is practically the same in the extract obtained in the cold as compared with that carried out at the boiling point of the solvent.

TABLE 2.

A. Extraction with alcohol followed by ether and then alcohol, at room temperature.

NO.	WEIGHT OF DRIED HEART MUSCLE	ALCOHOL EXTRACT	ETHER AND ALCOHOL EXTRACT	TOTAL EXTRACT	PERCENTAGE OF LIPINS IN TISSUE	PERCENTAGE OF PHOSPHOLIPIN IN THE LIPIN EXTRACT
	grams	grams	grams	grams		
1	32.2340	4.4970	1.6805	6.1775	19.16	43.57
2	45.1300	6.7365	2.0810	8.8175	19.54	42.98

B. Extraction with alcohol followed by ether and then alcohol in Soxhlet apparatus, at boiling point of solvent.

1	44.5830	6.0630	3.0365	9.0995	20.41	42.86
2	44.2490	6.1035	3.3055	9.4909	21.26	42.18

On comparing these extractions, we find that the amount of extract obtained at the boiling point of the solvent is about 1.0 per cent higher than that obtained in the cold, while the percentage of phospholipins contained in the extract is about the same in both cases. It may also be noted that extraction with ether followed by alcohol and then ether at room temperature gives about 2 per cent less extract when compared with the extract obtained by extraction with alcohol followed by ether and then alcohol at room temperature.

NOTE ON FOLIN'S MICROCHEMICAL METHOD FOR THE DETERMINATION OF UREA.

By JOSEPH C. BOCK.

(From the Nutrition Laboratory of the Carnegie Institution of Washington, Boston, Massachusetts.)

(Received for publication, February 25, 1913.)

The total nitrogen in urines as determined by the method of Folin and Farmer¹ can be obtained by determining the ammonia colorimetrically or by titration with $\frac{N}{100}$ or $\frac{N}{1000}$ alkali and acid. The urea nitrogen is usually determined colorimetrically,² but the somewhat shorter method of titration was tried in our laboratory. When the urea nitrogen is determined colorimetrically the results are very accurate, but when the titration is used the results obtained are too low. I therefore conducted some experiments to find the cause of this deficiency.

I started by making blank tests with different brands of sodium acetate. Seven grams of sodium acetate were put into a Jena test tube (200 mm. by 20 mm.), a porcelain shot (to prevent bumping), the heat indicator and 1 cc. of 50 per cent acetic acid were added and, in place of the urea solution, 1 cc. of ammonia-free water was used. The stopper with the tube, which acts as reflux condenser, was put in and the tube heated according to the method for ten minutes. After cooling for a short time, the condensing tube was rinsed with 5 cc. of water and, after adding 2 cc. of concentrated sodium hydroxide, air was blown for ten minutes through the apparatus and into an Erlenmeyer flask containing 10 cc. of $\frac{N}{100}$ hydrochloric acid and 40 cc. of water. The air current was of the strength used in all our ammonia and nitrogen determinations.

In each case I found that the amount of acid in the Erlenmeyer flask had increased, which would of course lower the amount of nitrogen. These tests were all made by cooling the tube to room

¹ Folin and Farmer: this *Journal*, xi, p. 493, 1912.

² Folin: this *Journal*, xi, p. 507, 1912.

temperature before blowing the air through. Another series of tests was made without cooling, the mixture in the tube having a temperature of about 45°. The table gives the excess of acid expressed in cubic centimeters of $\frac{N}{100}$ sodium hydroxide.

	SODIUM ACETATE BRAND		
	B. A.	J. T. B.	Ka
At room temperature....	0.75	0.60	0.6
	0.95	0.60	0.8
	0.65	0.70	0.6
	0.90	0.65	0.7
	1.05		
At 45°C.....		1.6	1.8
		1.7	1.4
		1.5	1.7

These results show that the increase in acidity is greater when the air is blown through the mixture when still hot, that is to say, at a temperature which the liquid will have when the adding of the water, exchange of stoppers, neutralizing, etc., is done in the shortest time possible.

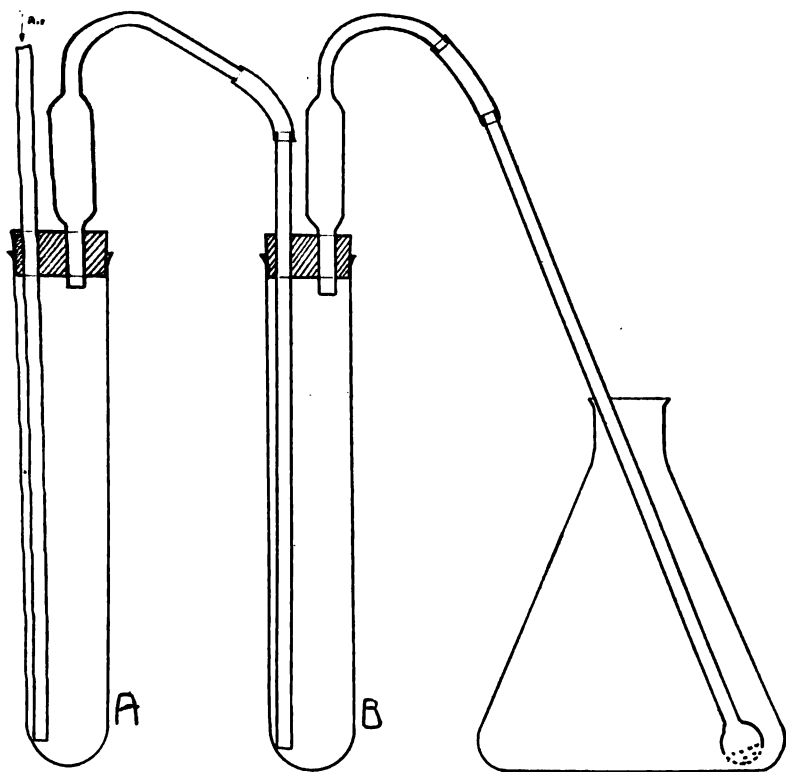
After the mixture is neutralized and cooled below room temperature, a strong smell of acetic acid in the tube is still noticeable. There is a measurable amount of acetic acid vapors standing in the tube which does not condense on cooling and is not neutralized by the sodium hydroxide. The following experiment will prove this statement.

After cooling and adding the water, the sodium hydroxide solution was added and, before blowing the air through the liquid, the air column containing the acetic acid vapors was removed by blowing a gentle current of air in the tube without disturbing the liquid. This could of course not be done in the case of an actual determination. The rest of the experiment was carried out as usual and the acidity was found to be less.

Increase in acidity after removing air column on top of liquid	0.30
	0.40
	0.20
	0.40
	0.35

After this I tried adding more than 2 cc. of sodium hydroxide. This decreases the acidity somewhat, but does not retain all the acid. Besides it makes the passing of air more difficult on account of the increased bulk of liquid in the tube.

A different arrangement of apparatus was therefore necessary. A second tube, *B*, exactly like the first one and fitted with a stopper with the same arrangement of tubes, was put between the tube *A* containing the mixture and the Erlenmeyer flask which holds the $\frac{N}{100}$ hydrochloric acid. This tube contained 3 cc. of 40–45 per cent sodium hydroxide, so that the air had to pass through the strong alkali before going into the standard solution.



It was necessary to pass the air current through for fifteen minutes instead of ten minutes as previously. The following results

show that with this arrangement all the acetic acid is retained and satisfactory results are obtained. The figures give the amount in cubic centimeters of urea nitrogen found.

The sample used was a solution of pure urea made up to contain 1 mgm. of nitrogen per cubic centimeter.

0.995	0.993	1.001
0.993	0.995	1.001

RESEARCHES ON PURINES.

ON 2-THIO-6,8-DIOXYPURINE AND 2,8-DITHIO-6-OXYPURINE. ON THE DESULPHURIZATION OF THIOPURINES. ON A NEW METHOD OF PREPARING XANTHINE.

NINTH PAPER.¹

BY CARL O. JOHNS AND ALBERT G. HOGAN,

(From the Sheffield Laboratory of Yale University.)

(Received for publication, February 25, 1913.)

Only one of the three dioxy-monothio-purines theoretically possible has been described, namely, 2,6-dioxy-8-thiopurine.² We find that 2-thio-6,8-dioxypurine (V) can easily be prepared in quantity by heating a mixture of 2-thio-4,5-diamino-6-oxypyrimidine (II)³ and urea. The reaction is very smooth, the yield being almost quantitative.

None of the three monoxy-dithio-purines required by theory has hitherto been described. We have also prepared one of these compounds, namely, 2,8-dithio-6-oxypurine (III). This purine was obtained in good yields by heating a mixture of 2-thio-4-5-diamino-6-oxypyrimidine and thiourea.

Some years ago Wheeler and Liddle⁴ found that when 2-thiouracil (X) was boiled in a solution of monochloroacetic acid the thio-pyrimidine was converted to uracil-2-thioglycollic acid (XI) which being unstable was consequently hydrolyzed to uracil (XII). Johnson, Pfau and Hodge⁵ have shown that thiohydantoins can readily be desulphurized by boiling them with an aqueous solution of chloroacetic acid. The thiopurines described in this paper form

¹ This *Journal*, xiv, p. 1, 1913.

² E. Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 445, 1898.

³ W. Traube: *Ann. d. Chem.* (Liebig), cccxxxi, p. 75, 1904.

⁴ Wheeler and Liddle: *Amer. Chem. Journ.*, xl, p. 552, 1908.

⁵ Johnson, Pfau and Hodge: *Journ. Amer. Chem. Soc.*, xxxiv, p. 1041, 1912.

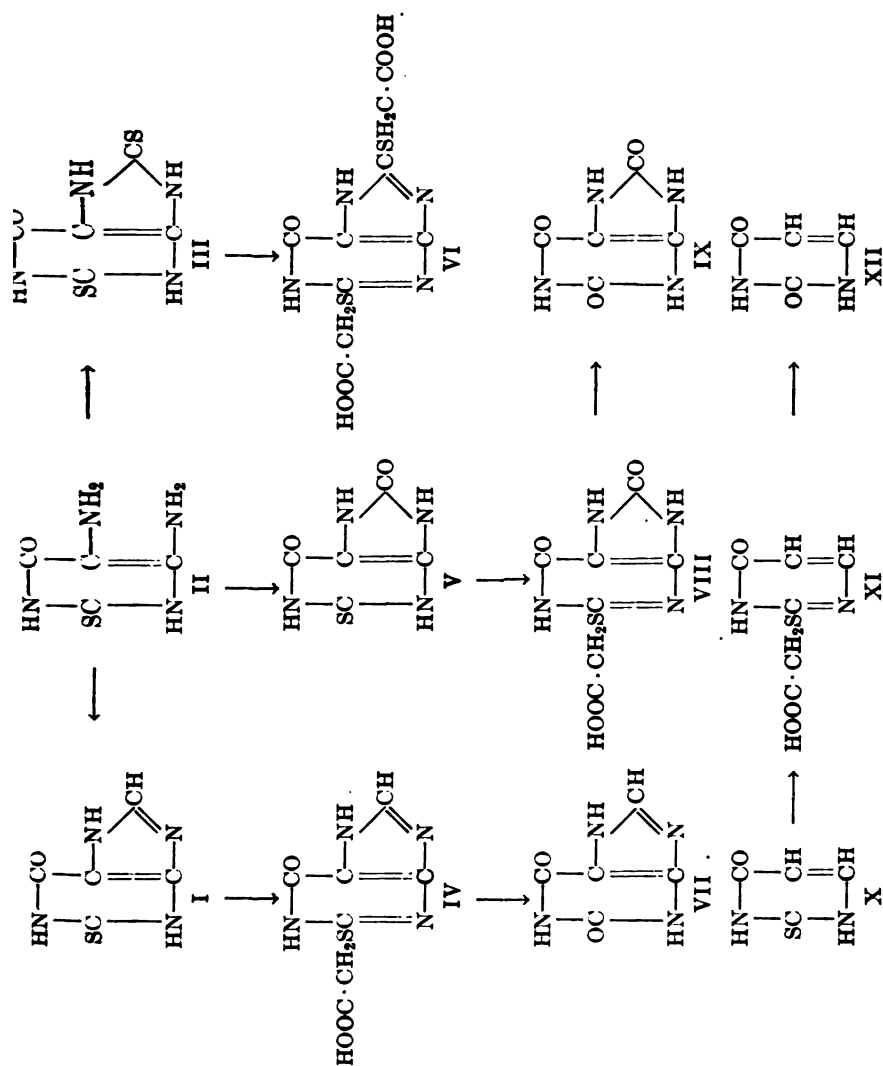
thioglycollic acid derivatives which are stable. Thus, hypoxanthine-2-thioglycollic acid (IV) and 6,8-dioxypurine-2-thioglycollic acid (VIII) could be boiled with water for hours without undergoing notable decomposition. On the other hand, when these compounds were boiled with 20 per cent hydrochloric acid, they were hydrolyzed and xanthine (VII) and uric acid (IX), respectively, were obtained. It was found that 6-oxypurine-2,8-dithioglycollic acid (VI) was not desulphurized by boiling for several hours with 20 per cent hydrochloric acid although a small quantity of a dioxypurine-monothioglycollic acid was obtained. This substance was probably 2,6-dioxypurine-8-thioglycollic acid, since, if 6,8-dioxypurine-2-thioglycollic acid had been formed it would have been hydrolyzed to uric acid by the action of the hydrochloric acid. The fact that 6-oxypurine-2,8-dithioglycollic acid is more stable than the monothioglycollic acid derivatives accords with an observation made by Wheeler and Liddle⁶ who found that pyrimidine-2,6-dithioglycollic acid was not converted to uracil even when warmed with hydrochloric acid.

Hitherto the best method of making xanthine has been by the action of nitrous acid on guanine. The xanthine prepared in this manner was highly colored and the color was very difficult to remove. Fischer⁷ used a mixture of fuming hydriodic acid and phosphonium iodide to remove the color from xanthine which had been prepared from guanine. The method described in the following pages gives almost colorless xanthine even without the use of an adsorbent.

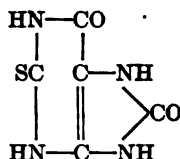
As 2-thiohypoxanthine (I) can be made in quantity and is easily converted to xanthine, as herein described, this procedure may be recommended as a method of preparing xanthine in quantity. The stability of the purine-thioglycollic acids renders it probable that these derivatives can be used for various synthetic purposes. These researches will be continued.

⁶ Wheeler and Liddle: *Amer. Chem. Journ.*, xl, p. 557, 1908.

⁷ E. Fischer: *Untersuchungen in der Puringruppe*, p. 267.

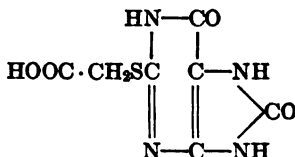


EXPERIMENTAL PART.

2-Thio-6,8-dioxypurine.

Five grams of 2-thio-4,5-diamino-6-oxypyrimidine^a and 5 grams of urea were pulverized together and the mixture was heated in an oil bath at 170–180°C. for an hour. This treatment gave a hard crust which was dissolved in hot dilute sodium hydroxide. After filtering off a trace of insoluble substance, the hot filtrate was acidified with acetic acid. A precipitate formed immediately. This consisted of minute crystals and weighed 5.4 grams or 93 per cent of the calculated weight. The crystals were very stable and did not melt at 310°C. They were soluble in about 500 parts of boiling water and almost insoluble in cold water. They did not dissolve in alcohol or benzene. They were moderately soluble in dilute ammonia and easily soluble in dilute sodium hydroxide. They gave a brilliant murexide reaction. An ammoniacal solution of the purine gave a precipitate on adding a few drops of silver nitrate solution. The substance was dried at 130°C. for analysis.

	Calculated for $\text{C}_4\text{H}_4\text{O}_2\text{N}_2\text{S}$	Found:	
		I	II
N.....	30.43	30.53	30.73

6,8-Dioxypurine-2-thioglycollic acid.

This compound could be obtained by boiling 2-thio-6,8-dioxypurine in an aqueous solution of monochloroacetic acid but owing to the slight solubility of the dry thiopurine it was prepared as follows:

^a W. Traube: *Ann. d. Chem.* (Liebig), cccxxxi, p. 75, 1904.

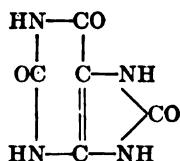
Two grams of 2-thio-6,8-dioxypurine were suspended in 150 cc. of hot water and just enough sodium hydroxide to effect solution was added. The purine was then reprecipitated in a finely divided state by adding acetic acid. To this mixture were then added 6 grams of monochloroacetic acid and on boiling for twenty minutes under a return condenser complete solution took place. This solution was evaporated to dryness, the residue was dissolved in dilute ammonia and on acidifying with acetic acid the glycollic acid derivative was obtained as a finely divided precipitate. This dissolved in about 100 parts of boiling water but was difficultly soluble in cold water and did not dissolve in alcohol or benzene. It did not have a melting point but decomposed at about 225°C. It gave a murexide reaction and its ammoniacal solution gave a precipitate on adding silver nitrate. The yield was quantitative. The substance was dried at 130°C. for analysis.

	Calculated for $C_7H_5O_4N_4S$	Found:
N	23.14	23.40

The ammonium salt of 6,8-dioxypurine-2-thioglycollic acid was obtained when the purine was dissolved in dilute ammonia and the solution was acidified with acetic acid.

	Calculated for $C_7H_5O_4N_4S$	I	Found: II
N	27.02	26.90	26.85

Uric acid.

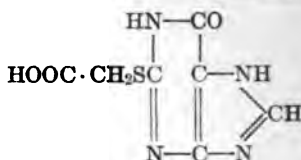


The ammonium salt of 6,8-dioxypurine-2-thioglycollic acid was dissolved in hot 20 per cent hydrochloric acid and the solution was boiled for two hours. Uric acid was obtained. The reaction was quantitative. Uric acid was also obtained from the free purine as follows: A solution of 6,8-dioxypurine-2-thioglycollic acid was prepared as previously described. An equal volume of concentrated hydrochloric acid was then added and the solution was

boiled under a reflux condenser for two hours. The mixture was evaporated to dryness and the residue was dissolved in dilute sodium hydroxide. When the hot solution was acidified with hydrochloric acid, uric acid was obtained in crystalline form. The yield was quantitative.

	Calculated for $C_4H_4O_4N_4$	Found:	
		I	II
N	33.33	33.10	33.20

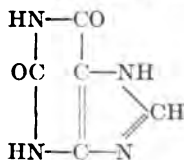
Hypoxanthine-2-thioglycollic acid.



Two grams of 2-thiohypoxanthine^a were suspended in 150 cc. of hot water and sufficient sodium hydroxide to produce solution was added. The thiohypoxanthine was then precipitated in a finely divided state by means of acetic acid. Six grams of monochloroacetic acid were added and the mixture was boiled under a return condenser. In about fifteen minutes the solution was clear and was then evaporated to dryness on a steam bath. The residue was washed with cold water to remove salts and the thioglycollic acid derivative was obtained. This was sparingly soluble in hot and difficultly soluble in cold water and insoluble in alcohol or benzene. It dissolved readily in ammonia or dilute sodium hydroxide. It decomposed at about 240°C. forming a violet colored substance. Its ammoniacal solution gave a precipitate with silver nitrate. The substance was dried at 130°C. for analysis.

	Calculated for $C_7H_6O_4N_4S$	Found:
N	24.77	24.60

2-6-Dioxypurine (Xanthine).



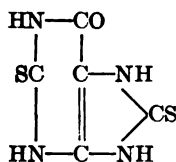
^a W. Traube: *Ann. d. Chem.* (Liebig), cccxxxi, p. 77, 1904.

One part of hypoxanthine-2-thioglycollic acid was dissolved in about 75 parts of hot 20 per cent hydrochloric acid and the solution was boiled under a reflux condenser for two hours. After evaporating to dryness the residue was taken up in hot dilute sodium hydroxide and the filtered solution was acidified with acetic acid. The xanthine which precipitated was almost colorless.

In order to prepare xanthine from 2-thiohypoxanthine it is unnecessary to isolate the intermediate thioglycollic acid derivative. When a solution of the latter compound has been prepared, as previously described, an equal volume of concentrated hydrochloric acid is added and the mixture is boiled for two or three hours and then evaporated to dryness. The yield of xanthine is almost quantitative.

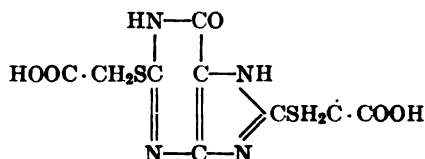
	Calculated for $C_5H_4O_4N_4$	Found:
N	36.84	36.71

2,8-Dithio-6-oxypurine.



Two grams of 2-thio-4,5-diamino-6-oxypyrimidine were pulverized together with 2 grams of thiourea and the mixture was heated in an oil bath at 180°C. for an hour. The reaction product was dissolved in hot dilute sodium hydroxide, the solution was filtered to remove a little insoluble material and the hot filtrate was acidified with acetic acid. A granular precipitate was obtained. This was purified by dissolving in dilute ammonia and reprecipitating with acetic acid. The dithiopurine was soluble in about 100 parts of boiling water but did not dissolve in alcohol or benzene. It did not melt at 310°C. It gave a murexide reaction and its ammoniacal solution gave a precipitate on the addition of silver nitrate.

	Calculated for $C_4H_4ON_4S_2$	Found:	
		I	II
N	28.00	27.77	27.50

6-Oxypurine-2,8-dithioglycollic acid.

One gram of 2,8-dithio-6-oxypurine was dissolved in 75 cc. of water containing sufficient sodium hydroxide to produce solution. The purine was then precipitated from the hot solution by adding acetic acid. Six grams of monochloracetic acid were added and the mixture was boiled until solution took place, whereupon it was evaporated to dryness on the steam bath. The residue was washed in a little cold water. The resulting dithioglycollic acid derivative was easily soluble in hot and moderately soluble in cold water and slightly soluble in hot alcohol. It decomposed with effervescence at about 240°C. It gave a murexide reaction and its ammoniacal solution gave a precipitate with silver nitrate. The substance was dried at 130°C. for analysis.

	Calculated for $\text{C}_7\text{H}_5\text{O}_4\text{N}_4\text{S}_2$	Found:
N	17.83	18.50

A portion of the 6-oxypurine-2,8-dithioglycollic acid was boiled with 20 per cent hydrochloric acid for several hours without the formation of uric acid. A difficultly soluble compound was obtained and this gave an analysis for a dioxypurine-monothioglycollic acid. This was probably 2,6-dioxypurine-8-thioglycollic acid since, if 6,8-dioxypurine-2-thioglycollic acid had been formed, it would have been hydrolyzed to uric acid. The substance was dried at 130°C. for analysis.

	Calculated for $\text{C}_7\text{H}_4\text{O}_4\text{N}_4\text{S}$	Found:
N	23.14	22.8%

RESEARCHES ON PYRIMIDINES: PYRIMIDINE-NUCLEOSIDES.

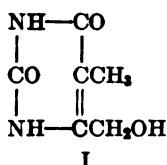
BY TREAT B. JOHNSON AND LEWIS H. CHERNOFF.

(SIXTY-FIRST PAPER.)

(Contributions from the Sheffield Laboratory of Yale University.)

(Received for publication, February 28, 1913.)

This paper is a contribution to our knowledge of the constitution of pyrimidine nucleosides. It is our first paper on this subject and includes a description of the synthesis and properties of the simplest nucleoside of thymine (I).



Through the investigations of Levene¹ and his co-workers our knowledge of the constitution of nucleic acids has been advanced to such a degree that we may now regard these interesting substances as composed of characteristic complexes designated by the term *nucleotides*.² The latter are compounds consisting of phosphoric acid conjugated with a complex composed of a carbohydrate and a purine or a pyrimidine. In other words, a nucleic acid may be a single nucleotide, as in the case of guanylic³ and

¹ Levene and Mandel: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 1905; Levene and Jacobs: *ibid.*, xlii, p. 2474; xlii, p. 2704; xliii, p. 3150; xlv, p. 1027; Levene and La Forge: *ibid.*, xliii, p. 3164; xlv, p. 608; Levene and Jacobs: this *Journal*, xii, pp. 411, 421; Mandel and Dunham: *ibid.*, xi, p. 85.

² Levene and Mandel: *loc. cit.*

³ Levene and Jacobs: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 2469; this *Journal*, xii, p. 421.

inosinic⁴ acids (mononucleotides), or be composed of several nucleotides as in the case of yeast nucleic acid. This acid or polynucleotide is a combination of four different nucleotides containing the two purines, guanine and adenine, and the pyrimidines, cytosine and uracil. This nucleotide structure is apparently common to all nucleic acids. Regarding the nature of the union of the individual nucleotides in polynucleotides we have no definite knowledge, but it seems very probable, from the evidence presented, that they are condensed in one molecule in the case of yeast nucleic acid.

The constitution of these nucleotides has also been partly elucidated by the work of these same investigators. It has been shown by Levene⁵ that these compounds may lead, by partial hydrolysis, to the formation of two types of complexes depending upon the experimental conditions employed. For example, it is possible to detach from a nucleotide phosphoric acid alone giving a simpler complex of a sugar and a purine or pyrimidine, viz., *nucleosides*,⁶ or to remove only the nitrogenous nucleus, leaving the phosphoric acid in combination with the carbohydrate. These results are significant and prove that the three constituents of a nucleotide are linked according to the following order—acid, sugar, base. Regarding the constitution of the nucleosides of yeast nucleic acid Levene and Jacobs⁷ write as follows: "Die organischen Komplexe der Hefenucleinsäure sind also in zwei Klassen einzuteilen: Die der Purinbasen, welche glykosidartige Verbindungen darstellen, und die der Pyrimidinbasen, deren Konstitution noch nicht ganz aufgeklärt ist."

Direct proof of the presence of these purine-carbohydrate and pyrimidine-carbohydrate combinations has been presented by the isolation of the pentose nucleosides, viz.: guanosine, adenosine, uridine and cytidine⁸ from yeast nucleic acid and of guanine-

⁴ Levene and Jacobs: *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 276; xlii, p. 335.

⁵ Levene and co-workers: *loc. cit.*

⁶ Levene and Jacobs: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 2475.

⁷ *Ibid.*, xlii, p. 1027.

⁸ Levene and co-workers: *loc. cit.*

hexoside⁹ from thymus nucleic acid. In chemical terms the nucleotides and their corresponding nucleosides may be expressed by the following empirical formulas:

<i>Nucleotides.</i>	<i>Nucleosides.</i>
O:P(OH) ₂ . C ₅ H ₃ O ₄ . C ₅ H ₄ N ₄ .	C ₅ H ₃ O ₄ . C ₅ H ₄ N ₄ .
II	Adenosine
O:P(OH) ₂ . C ₅ H ₃ O ₄ . C ₅ H ₄ ON ₄ .	III
Guanylic acid.	C ₅ H ₃ O ₄ . C ₅ H ₄ ON ₄ .
IV	Guanosine.
O:P(OH) ₂ . C ₅ H ₃ O ₄ . C ₅ H ₄ ON ₄ .	V
Inosinic acid.	C ₅ H ₃ O ₄ . C ₅ H ₄ ON ₄ .
VI	Inosine.
O:P(OH) ₂ . C ₅ H ₃ O ₄ . C ₅ H ₄ ON ₃ .	VII
Cytidine-nucleotide.	C ₅ H ₃ O ₄ . C ₅ H ₄ ON ₃ .
VIII	Cytidine.
O:P(OH) ₂ . C ₅ H ₃ O ₄ . C ₅ H ₄ O ₂ N ₃ .	IX
Uridine-nucleotide.	C ₅ H ₃ O ₄ . C ₅ H ₄ O ₂ N ₃ .
X	Uridine.
	XI

In their fifth paper on yeast nucleic acid entitled: "Die Struktur der Pyrimidin-Nucleoside" Levene and La Forge¹⁰ have presented conclusive evidence that the pyrimidine nucleosides, uridine and cytidine, are combinations of uracil and cytosine, respectively, with the pentose sugar ribose. In fact, the exact relationship of these two nucleosides was established by this observation, since it had already been shown that the pyrimidines are linked, in these two nucleosides, to the carbohydrate in a similar manner. This was established by the fact that cytidine is transformed by the action of nitrous acid into uridine.¹¹ Regarding the nature of this nucleoside union and the position substituted by the sugar in the pyrimidine ring, sufficient data have not been presented to enable us to express structurally the exact constitution of these compounds. Levene and La Forge¹² conclude, however, from good evidence, which we will not discuss in this paper, that this linking is of a glucosidic nature and that the carbohydrate may be joined

⁹ Levene and Jacobs: this *Journal*, xii, p. 377.

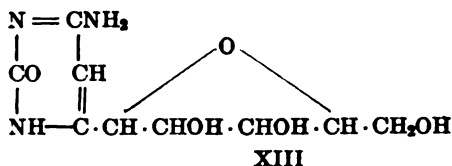
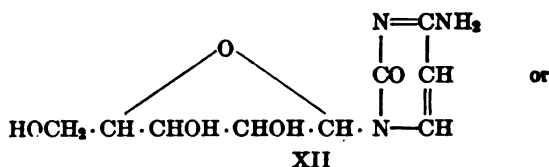
¹⁰ *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 608.

¹¹ Levene and Jacobs: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 1027.

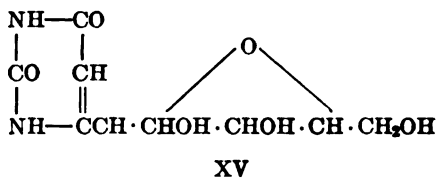
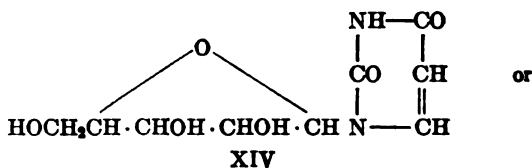
¹² *Loc. cit.*

to the pyrimidine in one of two positions, viz., the 3- or 4-position of the ring. If these assumptions be correct then the constitution of cytidine and uridine may be expressed by the following structural formulas:

Cytidine.



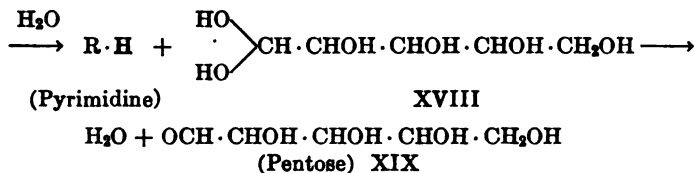
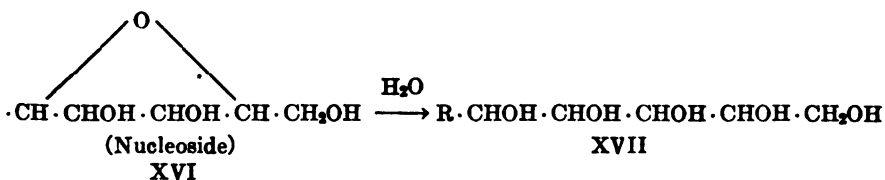
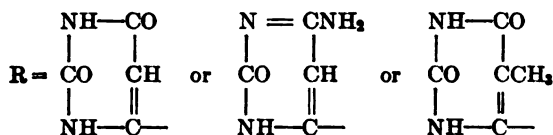
Uridine.



The remarkable stability of these substances, in the presence of hydrolytic agents, seems to indicate that the point of union of the carbohydrate is in position 4 of the ring, as represented by formulas (XIII) and (XV), rather than in position 3 as represented by formulas (XII) and (XIV). Whether the pyrimidine-nucleosides (hexosides) of thymus nucleic acid correspond in constitution to the nucleosides under discussion, has not been established.

The recent work of Levene and Jacobs¹³ on thymus nucleic acid seems to indicate that the determination of the constitution of this acid is a far more complex problem than that presented by yeast nucleic acid.

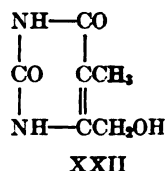
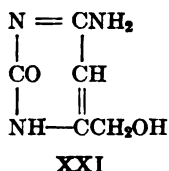
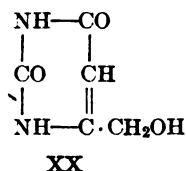
The synthetical work now in progress and also that discussed in this paper, has developed from the assumption that the pyrimidines, in their corresponding nucleosides, are linked to the carbohydrates at position 4 and that this linkage is between two carbon atoms as represented by formulas (XIII) and (XV). A nucleoside may be considered, therefore, as an addition-product of a pyrimidine and a sugar. The formation of ribose from such a complex, by hydrolysis, would then involve, theoretically, two distinct changes, viz.: a rupture of the furane ring forming the glucoside (XVII) and finally a cleavage of the carbohydrate (XIX) from the pyrimidine. These various changes may be expressed as follows:



Judging from what has already been observed in this laboratory, it would be predicted that a union of this character would be extremely stable and very resistant to the action of hydrolytic agents.

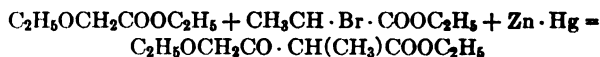
¹³ This *Journal*, xii, p. 411.

We can conceive, therefore, of an homologous series of these pyrimidine-nucleosides, each differing from its next member in the series by $-\text{CHOH}$. The physical properties of members of such a series would be expected to undergo a gradual change while, on the other hand, the chemical properties would be the same. Consequently if we successively removed a $-\text{CHOH}$ from the chain of the sugar molecule we would finally obtain the prototype of the series or the simplest nucleoside of this type. It would still retain the same glucosidic linking. The three pyrimidines, which would result by this process, are represented by formulas (XX), (XXI) and (XXII).



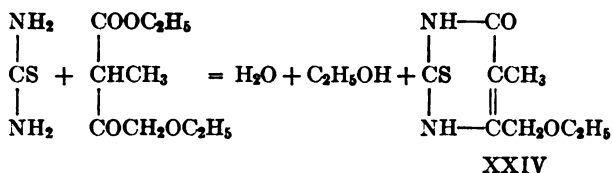
We have absolutely no knowledge of the chemical properties of hydroxypyrimidines of this type and it was not until recently that the writer was able to undertake their investigation. We are now able, however, to contribute data regarding their chemical behavior, which we believe to be of great biochemical interest. We shall now discuss the synthesis of the simplest nucleoside of thymine, viz.: 2,6-dioxy-4-hydroxymethyl-5-methylpyrimidine (XXII).

The new ketone ester, ethyl methylethoxyacetoacetate (XXIII) was first prepared by the condensation of ethyl α -bromopropionate with ethyl ethoxyacetate in the presence of zinc-amalgam.

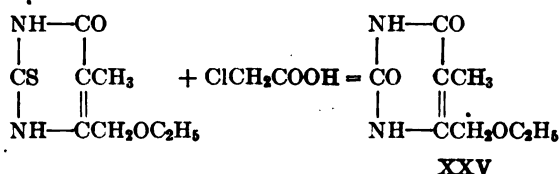


XXIII

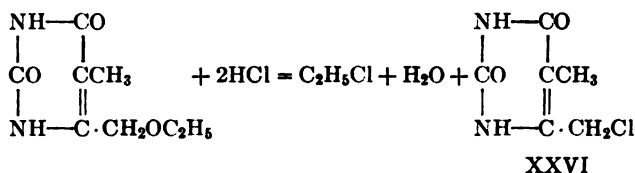
This ester (XXIII) was then digested in alcohol with thiourea and sodium ethylate when a pyrimidine condensation was effected and 2-thio-4-ethoxymethyl-5-methyl-6-oxypyrimidine (XXIV) was formed. This reaction is expressed by the following equation:



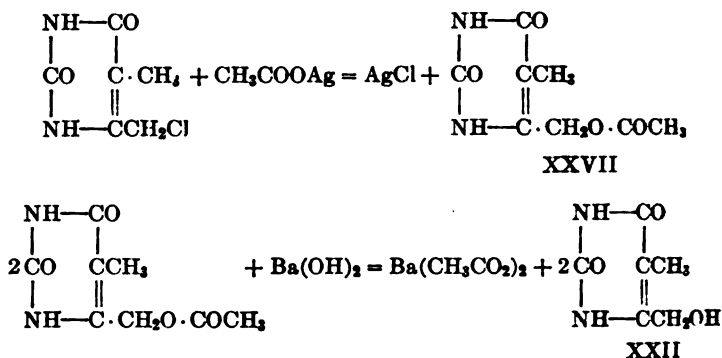
This thiopyrimidine (XXIV) was then digested with chloracetic acid when it was desulphurized practically quantitatively and the corresponding oxypyrimidine (XXV) was formed.



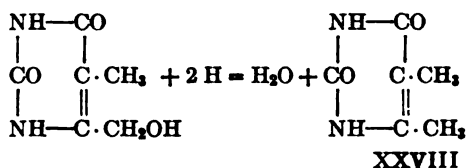
This ethoxypyrimidine (XXV) was then heated with concentrated hydrochloric acid. By means of this reagent the ethyl group was detached in the form of ethylchloride and the pyrimidine was converted smoothly into the chlormethylpyrimidine (XXVI). This interesting change is represented by the following equation:



The halogen in this pyrimidine (XXVI) is very reactive and is easily removed by alkaline hydrolysis. For example: the pyrimidine was transformed, almost quantitatively, into the corresponding acetate (XXVII) when digested in aqueous solution with the required amount of silver acetate. This pyrimidine possessed characteristic properties and underwent a smooth hydrolysis, when digested with barium hydroxide solution, forming the corresponding alcohol (nucleoside) (XXII). These final changes in this synthesis are represented as follows:



The constitution of this nucleoside (XXII) was established by the fact that it was transformed quantitatively into 2,6-dioxy-4-5-dimethylpyrimidine¹⁴ (XXVIII) by reduction with hydriodic



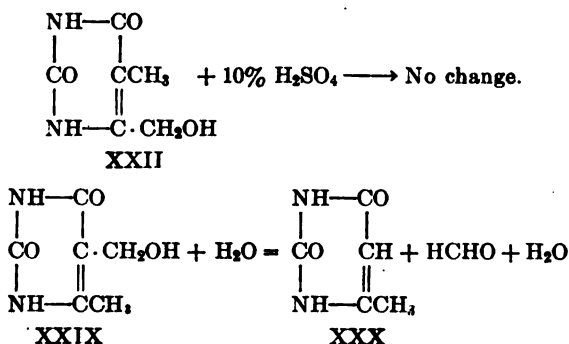
acid and red phosphorus. Starting with the two aliphatic esters, ethyl α -bromopropionate and ethyl ethoxyacetate, our synthesis therefore involves six operations, and in every step, except the first, the yields are excellent. We are now investigating this unsatisfactory step in order to determine the cause of the low yield.

We now find that this simple nucleoside of thymine (XXII) is extremely stable in the presence of acids and does not undergo hydrolysis with formation of thymine and formaldehyde when heated with sulphuric acid. It has been shown in the papers by Levene and his co-workers¹⁵ that the carbohydrate is cleaved from the pyrimidine-nucleosides by heating with 10 per cent sulphuric acid at 125°. Our pyrimidine was recovered unaltered after heating with sulphuric acid, of this same strength, for three hours at 125–130°. This result is all the more interesting since

¹⁴ Schlenker: *Ber. d. deutsch. chem. Gesellsch.*, xxxiv, p. 2812; Wheeler and Merriam: *Amer. Chem. Journ.*, xxix, p. 488.

¹⁵ *Loc cit.*

Kircher¹⁶ has recently shown that the isomeric pyrimidine, 2,6-dioxy-4-methyl-5-hydroxymethylpyrimidine (XXIX) is transformed quantitatively into 4-methyluracil (XXX) and formaldehyde simply by heating in aqueous solution. From a chemical standpoint these results are extremely interesting and very significant. Whether the linking between the carbohydrate complex and the pyrimidine at position 4 will become more unstable as we increase the length of the sugar chain, must be decided by further investigations. We shall endeavor to develop methods of synthesizing some of these higher homologues. The corresponding hydroxy derivatives of uracil (XX) and cytosine (XXI) will also be investigated.



EXPERIMENTAL PART.

*Ethyl methylethoxyacetoacetate.*¹⁷



This interesting β -ketone ester was prepared by condensing ethyl α -bromopropionate with ethyl ethoxyacetate by means of zinc-amalgam. The method of procedure was essentially as follows: Molecular proportions of the bromopropionate (84.4 grams)

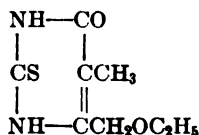
¹⁶ *Ann. d. Chem.* (Liebig), ccclxxxv, p. 293.

¹⁷ This new method of synthesis, which we have employed for the preparation of this ester, has been applied successfully in several other cases. The reaction is being investigated and the results of the investigation will be published in future papers. We are also using zinc-amalgam as a reagent in other lines of investigation and have obtained interesting results, which we soon hope to be able to present for publication. (T. B. Johnson.)

and the acetate (56.0 grams) were placed in a dry flask and 40.8 grams of dry amalgamated zinc suspended in the liquid. The flask was then connected with a return condenser and finally heated on the steam bath. At first there was no evidence of any reaction, but after warming a few minutes a violent reaction began and became so vigorous that it was necessary to plunge the flask into ice water at intervals to avoid too great heat. After the violent reaction was over the flask was then heated on the steam bath for about twelve hours in order to thoroughly complete the reaction. We obtained a dark brown, syrupy fluid. This was then transferred to a separatory funnel and shaken with an excess of water when we obtained a heavy precipitate, which was immediately dissolved by addition of cold dilute hydrochloric acid. We obtained in this manner a transparent red oil, which was separated from the acid solution and finally dissolved in ether. This ether solution was then thoroughly cooled with crushed ice and washed repeatedly with a cold, dilute solution of sodium hydroxide. The β -ketone ester was removed by this treatment and the alkaline solutions finally combined and acidified (cold) with cold, dilute hydrochloric acid. The ketone ester separated at once and was dissolved in ether. After thorough drying over anhydrous calcium chloride the ether was removed and the ester purified by distillation under diminished pressure. It practically all distilled at 116° at 24 mm. The yield of purified material was 7.5 grams. Molecular weight determination by the ebulliscopic method:

I. 0.6731 gram substance in 15.56 grams of benzene gave $\Delta_b = .619^\circ$.		
	Calculated for	Found:
	$C_8H_{10}O_4$:	
Molecular Weight.....	188	186

2-Thio-4-ethoxymethyl-5-methyl-6-oxypyrimidine.

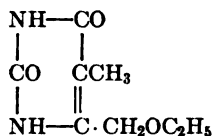


This pyrimidine was formed by condensation of the preceding β -ketone ester with thiourea in the presence of sodium ethylate. The following proportions were used: 7.4 grams of the ketone

ester, 3.0 grams of thiourea and 1.8 grams of metallic sodium. The sodium was dissolved in a small volume of absolute alcohol, the thiourea and ketone ester dissolved in the solution and the mixture then digested on the steam bath for about four hours. The sodium salt of the pyrimidine began to form almost immediately on heating, and deposited as a brown powder. After completion of the reaction the alcohol was then evaporated and the residue dissolved in a small volume of hot water and the solution filtered. On acidifying this solution (cold) with glacial acetic acid the pyrimidine separated at once in a crystalline condition. It was purified by crystallization from boiling 95 per cent alcohol and separated, on cooling, in hexagonal tables, which melted at 191–192° to a clear oil without decomposition. The yield of purified pyrimidine was 2.3 grams. The pyrimidine is very soluble in hot water and hot alcohol and difficultly soluble in cold.

NITROGEN DETERMINATION (Kjeldahl):

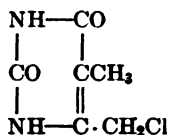
	Calculated for $C_6H_{12}O_2N_2S$:	Found:
N.....	13.86	13.90

2,6-Dioxy-4-ethoxymethyl-5-methylpyrimidine.

One and three-tenths grams of 2-thio-4-ethoxymethyl-5-methyl-6-oxypyrimidine and two molecular proportions of chloracetic acid (1.1 grams) were dissolved in 30 cc. of water and the solution boiled for five hours. The solution was then allowed to cool slowly when this pyrimidine separated in beautiful, arborescent crystals. The compound was purified by crystallization from hot water and melted at 220° to a clear oil. It is soluble in hot alcohol. The yield was 1.1 grams.

NITROGEN DETERMINATION (Kjeldahl):

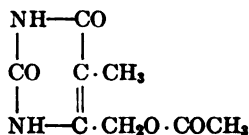
	Calculated for $C_6H_{12}O_2N_2S$:	Found:
N.....	15.05	15.02

2,6-Dioxy-4-chlormethyl-5-methylpyrimidine.

This chlorpyrimidine was prepared by heating the preceding pyrimidine with hydrochloric acid. One gram of the ethoxy-pyrimidine and 20-cc. of concentrated hydrochloric acid were heated in a bomb tube for three hours at 125–130°. When the tube was opened ethyl chloride was identified and a yellow solution was obtained. This was then concentrated on the steam bath and cooled when this chlorpyrimidine separated in the form of plates, which melted at 243° to a clear red oil. On evaporating the filtrate to dryness more of the same compound was obtained. The pyrimidine was purified by crystallization from boiling water and separated, on cooling, in stout prismatic crystals or blocks which melted at 244–245° to a clear oil without decomposition. It gave a strong test for chlorine. The dust from this pyrimidine irritates the nose causing violent sneezing and finally a severe headache. The yield was excellent.

NITROGEN DETERMINATION (Kjeldahl):

	Calculated for $\text{C}_6\text{H}_7\text{O}_2\text{N}_2\text{Cl}$:	Found:
N.....	16.04	15.81

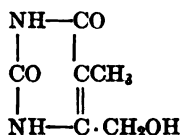
The acetate of 2-6-dioxy-4-hydroxymethyl-5-methylpyrimidine.

Two and four-tenths grams of pure silver acetate were dissolved in about 200 cc. of hot water and 2.086 grams of the above chlorpyrimidine added to the hot solution. There was an immediate reaction, the pyrimidine dissolved and silver chloride deposited. This solution was finally boiled for two hours in order to complete the reaction. After filtering from silver chloride the solution was then evaporated to complete dryness and the residue dissolved

again in hot water and the slight amount of silver salt in solution decomposed by treatment with hydrogen sulphide. After digesting with bone coal the solution was then filtered and concentrated to a small volume. On cooling, the acetylpyrimidine separated in minute crystals. It was difficultly soluble in hot water and cold 95 per cent alcohol. It was purified by crystallization from 95 per cent alcohol and deposited in microscopic, corpuscular crystals, which melted at 260–261° with effervescence. The yield of purified material was about 1.5 grams.

NITROGEN DETERMINATION (Kjeldahl):

	Calculated for $C_6H_9O_4N_2$	Found:
N.....	14.14	14.34

2,6-Dioxy-4-hydroxymethyl-5-methylpyrimidine.

This interesting pyrimidine was obtained by saponification of the above acetate. Five grams of crystallized barium hydroxide and one gram of the acetylpyrimidine were dissolved in the least possible volume of hot water and the solution boiled for one hour. The solution was then saturated with carbon dioxide gas in order to precipitate the barium as carbonate, and the solution finally filtered. The solution was then evaporated to dryness when the crude hydroxypyrimidine was obtained as an amber colored crystalline residue. This was purified by crystallization from hot water and separated, on cooling, in distorted needles, which melted at 224–225° with decomposition.

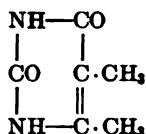
NITROGEN DETERMINATION (Kjeldahl):

	Calculated for $C_6H_9O_4N_2$	Found:
N.....	17.94	17.82

An attempt to convert this pyrimidine into thymine by hydrolysis with 10 per cent sulphuric acid was unsuccessful. One half of a gram of the pyrimidine was suspended in 25 cc. of 10 per cent sulphuric acid and then heated in a bomb tube for three hours at

120–130°. When the tube was opened there was no pressure and no evidence that the pyrimidine had undergone any change. The sulphuric acid was exactly precipitated as barium sulphate by addition of the required amount of barium hydroxide, and the barium-free filtrate then evaporated to dryness. We obtained a crystalline substance, which was purified by crystallization from hot water. It separated in the form of distorted prisms which melted at 223–224° with effervescence. It did not resemble thymine in any of its properties and was identified as the unaltered hydroxypyrimidine. We recovered all of the pyrimidine and used part of it for the following experiment:

The conversion of 2,6-dioxy-4-hydroxymethyl-5-methylpyrimidine into 2,6-dioxy-4,5-dimethylpyrimidine.



One-tenth of a gram of the hydroxypyrimidine was dissolved in 5 cc. of hydriodic acid (sp. gr. 1.7) and the solution, after the addition of a few milligrams of red phosphorus, was boiled for four hours. After cooling, the solution was then diluted with water, filtered and then an excess of dry silver carbonate stirred into the solution in order to remove all the iodine, hydriodic and phosphoric acids. After filtering, the excess of silver was then precipitated as sulphide and the solution then concentrated on the steam bath and finally allowed to cool. Dimethyluracil separated in the form of prismatic crystals, which melted at 296° when heated rapidly. If heated slowly the substance melted at 292–294° with partial decomposition. A mixture of this compound with 4,5-dimethyluracil¹⁸ melted at exactly the same temperature.

¹⁸ Schlenker: *loc. cit.*; Wheeler and Merriam: *loc. cit.*

STUDIES ON THE INTERMEDIARY METABOLISM OF AMINO-ACIDS.

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(From the Herter Laboratory, New York.)

(Received for publication, March 1, 1913.)

The object of the following paper is to record experiments concerning the intermediary metabolism of the amino-acids derived from proteins. By utilizing the method of liver perfusion, Embden and others have shown that certain amino-acids, particularly tyrosine and phenylalanine, may give rise to acetoacetic acid. Confirming these results, Baer and Blum have been able to demonstrate an increased excretion of acetoacetic acid and β -hydroxybutyric acid when phenylalanine and tyrosine are given to human diabetics. On the other hand, by employing glycosuric animals, rendered diabetic by pancreas extirpation or by phlorhizin administration, other amino-acids have been shown to be capable of furnishing glucose (Knopf, Glässner and Pick, Embden, Lusk, Ringer and others).¹

The value of these methods of investigation can hardly be questioned, although the interpretation of the results is often difficult. The formation of either acetoacetic acid or glucose from amino-acid can only take place as the result of intricate molecular rearrangements and much work is necessary before an adequate analysis of the reactions can be undertaken. It is of course open to question as to how closely the normal path of catabolism resembles that observed under abnormal conditions, but it may be safely asserted that an accurate knowledge of what may happen to a food substance under any conditions, no matter how far removed from normal, is likely to be helpful in gradually filling in the intricate mosaic of metabolic reactions.

¹ For the literature of the subject, the reader is referred to the article on Phlorhizinglukosurie by Lusk: *Ergeb. d. Physiol.*, xii, p. 315, 1912.

At the present time, by use of the methods referred to, we know that phenylalanine and tyrosine yield acetoacetic acid freely, leucine less readily, while its formation from isoleucine and histidine is somewhat doubtful. On the other hand, glycine, alanine, aspartic and glutamic acids are known to be capable of leading to sugar synthesis in the glycosuric organism. It would seem, therefore, as if a relatively sharp separation might be made between the amino-acids capable of either acetoacetic acid or glucose formation.

It seemed very desirable that these investigations should be extended. Accordingly, the behavior in the glycosuric animal of all the other amino-acids known to occur in proteins has been investigated and also the effect of a number of them on perfusion through a surviving liver.

It has been found that serine, cysteine, proline², ornithine and arginine are all capable of yielding large amounts of sugar when given to glycosuric dogs. Valine³, leucine, isoleucine, lysine, histidine, phenylalanine and tryptophane yield relatively little or no sugar. Ornithine, lysine, arginine, proline, tryptophane and di-iodotyrosine do not yield acetoacetic acid in marked amounts when added to blood perfusing a dog's liver.

As will be seen from the experimental details, it is difficult to definitely state that administration of an amino-acid yields absolutely no glucose, but it is relatively easy to distinguish between those that yield large amounts and those which yield little or none.

It seems not unlikely that the administration to a glycosuric animal of relatively large amounts of an amino-acid which in itself is not convertible into glucose may however lead to an apparent small glucose excretion through its mass action in displacing other amino-acids. The writer is therefore unwilling to attach much significance to results leading to an apparent increased glucose excretion of less than 20 per cent of the amino-acid given.

By combining the new results with those previously obtained, it would appear that certain generalizations are possible. The following table represents the collected data.

² The results in the case of proline have been recently published: *this Journal*, xiii, p. 513, 1913.

³ α -Hydroxyisovaleric acid under similar conditions yields little or no glucose.

SUBSTANCE	INCREASED GLUCOSE EXCRETION WHEN GIVEN TO GLYCOSURIC DOG*	INCREASED ACETOACETIC ACID WHEN PERFUSED THROUGH SURVIVING LIVER
Glycine.....	+	—
Alanine.....	+	—
Serine.....	+	—
Cysteine.....	+	—
Aspartic Acid.....	+	—
Glutamic Acid.....	+	—
Ornithine.....	+	—
Proline.....	+	—
Valine.....	—	—
Leucine.....	—	+
Isoleucine.....	—	?
Lysine.....	—	—
Arginine.....	+	—
Histidine†.....	—	+†
Phenylalanine.....	—	+
Tyrosine.....	—	+
Di-iodotyrosine.....	—	—
Tryptophane.....	—	—

* Only those amino-acids which yield relatively large amounts of glucose are recorded as positive. Doubtful cases are recorded as negative.

† See p. 328.

‡ The increased acetoacetic acid is probably not directly derived from histidine (p. 328).

The following conclusions may be tentatively drawn:

1. The amino-acids derived from proteins which may yield glucose freely in the glycosuric organism are all those containing two, three, four and five carbon atoms, except valine.

2. Arginine is the only amino-acid with more than five carbon atoms which may furnish glucose freely, and in this case the sugar evidently comes from the ornithine moiety with five carbon atoms, into which it may be converted by the action of arginase.

3. All the straight-chain amino-acids yield sugar with the exception of lysine.

4. The amino-acids with branched chains including valine, leucine and isoleucine furnish little or possibly no sugar.

5. Proline is the only cyclic amino-acid yielding much glucose. Undoubtedly opening of the ring is the first step in its breakdown. None of the aromatic amino-acids yields glucose in considerable amount.

6. The close structural relations between ornithine, (arginine), proline and glutamic acids, all of which yield approximately equiv-

alent amounts of glucose, would indicate that their catabolic paths may be similar.

7. Certain amino-acids, including tryptophane, lysine and valine, yield neither acetoacetic acid nor glucose in considerable amount.

8. The fact that, while alanine and serine yield large amounts of glucose in the glycosuric animal, phenylalanine, tyrosine and tryptophane, all of which contain an alanine side-chain, do not, is clearly in harmony with the view that the side-chain containing three carbon atoms of these amino-acids is broken up. This may be regarded as evidence in support of the writer's view as to the mechanism of acetoacetic acid formation from phenylalanine and tyrosine, in which it was suggested that two of the four carbon atoms of acetoacetic acid were derived from the side-chain and two from the nucleus.

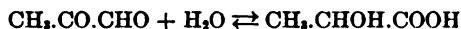
In conclusion, a few words may be said upon the possible mechanism of glucose formation from amino-acids, duly recognizing the fragmentary character of the evidence. The fact that, according to the careful experiments of Lusk and Ringer,⁴ glycine and alanine are quantitatively converted into glucose in the glycosuric organism would seem to inevitably point to the occurrence of a mechanism in the body for the reduction of the carboxyl group. Without such a change the fairly direct conversion of glycine and alanine into glucose is of course inconceivable; but at first sight the possibility of such a reaction is hard to picture.

The removal of the carboxyl group by a process of α -oxidation with formation of a lower aldehyde is incompatible with the quantitative glucose formation.

In searching for some more likely scheme, reference may be made to a new type of hydrolysis by an enzyme recently observed by Dudley and the writer. It was found that α -ketonic aldehydes, such as methyl glyoxal or phenyl glyoxal were converted into the corresponding α -hydroxyacids, namely, lactic and mandelic acids, with remarkable speed. If some such type of reaction should prove to be reversible, a solution might be found to the difficult problem of the mechanism of the reduction of the carboxyl group of alanine and similar acids.⁵

⁴ *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

⁵ Since the above was written Dudley and the writer have succeeded in showing the reversibility of this reaction *in vitro*.



The ready formation of methyl glyoxal from sugar *in vitro*, together with the fact that lactic acid as shown by Lusk is so readily converted into glucose in the diabetic organism, would lend support to the hypothesis of the intermediate formation of lactic acid and methyl glyoxal in the synthesis of glucose in the glycosuric animal. Moreover, the fact that both *d*- and *l*-alanine and probably *d*- and *l*-lactic acid are quantitatively converted into dextro-rotatory glucose would lead to the inference that the asymmetry of the α -carbon atoms in alanine and lactic acid is lost in the process of glucose synthesis. The intermediate formation of the methyl glyoxal would furnish an adequate explanation of this change since, on reconversion into sugar or lactic acid, asymmetry of the carbon atoms would be regained by a process of asymmetric synthesis.

Ringer and Lusk have shown that the amount of glucose obtainable from aspartic and glutamic acids in the glycosuric animal corresponds to about that derivable from three of the carbon atoms in each acid. They picture the hypothetical conversion of aspartic acid into hydracrylic acid and of glutamic acid into glyceric acid. To the writer it appears more probable that aspartic acid may yield either alanine or lactic acid. Some analogy for the removal of carbon dioxide from aspartic acid is found in its reduction to propionic acid by heating with hydriodic acid. The conversion of cysteine into taurine may also be cited as an example of the removal of the carboxyl group from an amino-acid.

The formation of a serine or alanine nucleus from glutamic acid would seem intelligible on the basis of β -oxidation as occurring in fatty acids, for glutamic acid is relatively a strong acid. What is true of glutamic acid may hold for proline and ornithine.

Finally, the fact that the amino-acids with branched chains do not readily form glucose in the glycosuric organism may be referred to the difficulty of lactic acid formation with its straight chain of three carbon atoms.

EXPERIMENTAL.

The experimental conditions were essentially those adopted in previous work.⁶ Phlorhizin (1 gram) suspended in olive oil was given once daily following Coolen's method.⁷ Urine was collected in twelve-hour periods with careful washing of the bladder. Glucose was estimated gravimetrically after polarimetric control. Acetoacetic acid and acetone were jointly estimated by distillation and titration with iodine in the customary manner. An approximate estimate of "extra glucose" was made by deducting the nitrogen of the amino-acid given from the total nitrogen of amino-acid and succeeding period. This amount multiplied by the dominant G:N⁸ ratio gives the endogenous glucose, which deducted from the total glucose excretion of the two periods gives the extra glucose. The method of calculation is no doubt rough, and depends upon the assumption that the whole of the nitrogen of the amino-acid administered is promptly excreted but it appears to be the best available.

The liver perfusions were carried out as in previous publications. The results are arranged in tabular form at the end of the paper.

Serine was obtained by Leuch's synthesis from chloroacetal. 100 grams of chloroacetal gave 14 grams of recrystallized serine. The serine was dissolved in water and injected subcutaneously.

Cysteine. Cystine was prepared from hair by Folin's method and then reduced to cysteine by means of tin and hydrochloric acid. The cystine (1 part) was dissolved in 10 parts of 20 per cent hydrochloric acid and granulated tin (1 part) added. After digestion on the water bath, the mixture was evaporated *in vacuo* to remove hydrochloric acid. The residue was then dissolved in water, filtered and the tin quantitatively removed with sulphuretted hydrogen. The filtrate was then evaporated to complete dryness *in vacuo* and the residue consisting of cysteine hydrochloride partly neutralized with sodium bicarbonate before injection.

In the second experiment the sulphur excretion was followed. I am indebted to Dr. Dudley for the analyses:

⁶ This *Journal*, ix, p. 146, 1911; xiv, p. 513, 1913.

⁷ *Arch. de pharmacodynamie*, i, p. 267, 1895.

⁸ The term G:N has been substituted for the more generally employed D:N. There seems no reason for retaining the term "dextrose" which has been long abandoned by organic chemists.

PERIOD	TOTAL S.	SULPHATE S.
II.	0.625	0.490
III.	1.397	1.308
IV.	1.164	1.050
V.	1.122	0.773

The sulphur excretion, principally in the form of sulphates, is relatively very slow.

Valine was obtained by the action of ammonia on α -bromisovaleric acid (Slimmer). The inactive acid was employed and was given by subcutaneous injection dissolved in warm water. It will be noted that there is no clear evidence of glucose or acetoacetic acid formation from valine. The excretion of acetoacetic acid was distinctly lowered on giving valine.

α -Hydroxyisovaleric acid. The acid was obtained by boiling α -bromisovaleric acid with calcium carbonate, followed by extraction of the acidified solution with ether. The acid crystallized on evaporation of the ether and was dried on a porous plate. Less than a gram of "extra glucose" followed the subcutaneous administration of the sodium salt from 15 grams of the acid.

Isoleucine. The amino-acid was obtained synthetically by Ehrlich's excellent method. The secondary butyl alcohol obtained by the reduction of methyl-ethyl-ketone was converted into the iodide since this reacts more smoothly with sodium malonic ester than does the bromide.

Leucine. A single experiment was carried out to supplement Halsey's work. Fifteen grams of inactive synthetic leucine led to an apparent excretion of 4 grams of "extra glucose."

Ornithine. The base was obtained by the action of arginase upon pure arginine followed by precipitation with phosphotungstic acid from dilute solution. The neutral acetate was used for injection.

Lysine, arginine. The bases were prepared by Kossel's methods. The neutral acetates were used for injection.

An attempt was made to estimate the amount of bases excreted unchanged in the urine by determining the amount of nitrogen precipitable by phosphotungstic acid after removal of ammonia. The results are recorded below:

	PERIOD	EXPERIMENT I	EXPERIMENT II
Arginine.....	II	0.068	0.052
	III	0.278	0.187
	IV	0.111	0.071
Lysine.....	II	0.052	0.190
	III	0.226	0.782
	IV	0.106	0.171

It is clear that in every case a small but definite rise in the basic nitrogen of the urine followed the injection of arginine and lysine. In the second lysine experiment, it amounted to about one-fifth of the nitrogen contained in the lysine given. In the other cases it was materially less.

Histidine. The crystalline hydrochloride was prepared by Fränkel's method in considerable quantity. In the earlier experiments, the hydrochloride was decomposed by an equivalent of sodium bicarbonate and injected intravenously. A minimal amount of ether was given to the dog as anaesthetic during the exposure of the vein. Under these conditions a marked increase in glucose output followed the administration of histidine, but later experience would indicate that at least some of this glucose did not originate from the base but was due to the anaesthetic and to metabolic disturbance following the intravenous injection. Therefore these results have been discarded. Later experiments, in which neutral histidine acetate, free from sodium chloride, was administered subcutaneously, showed in every case a slight increase in glucose output except in the case of one animal that had extraordinarily low sugar output throughout the experiment. A final decision, as to whether histidine really yields glucose in the diabetic animal, does not seem possible at present. Examination of the urines showed that from 2 to 7 per cent of the histidine was excreted unchanged. It will be noted that no regular increase in acetoacetic acid excretion was observed so that it appears unlikely that the slightly increased acetoacetic acid formation found on perfusing a surviving liver with blood containing histidine comes directly from the histidine. Histidine and lysine salts appear to be injurious to the perfused liver as judged by its rather congested and dark-colored appearance.

Phenylalanine. The synthetic acid was employed. No homogentisic acid was detected in the urine. An increased excretion of acetoacetic acid and β -hydroxybutyric acid was evident.

Tyrosine. The active amino-acid from casein was used. No increase in glucose excretion but a distinct increase in acetoacetic acid was observed, thus confirming the results of Lusk and Ringer.

Tryptophane was prepared by Hopkins and Cole's method. A slight increase in glucose excretion was observed but no acetoacetic acid formation. About three grams of kynurenic acid were separated from the urine by simple acidification with sulphuric acid. An attempt to estimate unchanged tryptophane in the urine by means of precipitation with mercuric sulphate after removal of kynurenic acid indicated the presence of about 3 grams of the amino-acid.

The metabolism of tryptophane in the glycosuric animal appears to resemble closely its behavior in the normal animal.

TABLE I.

SUBSTANCE	NITROGEN OF SUBSTANCE	WEIGHT OF DOG	PERIOD	TOTAL NITROGEN	GLUCOSE	C	N	EXTRA GLUCOSE	ACETOACETIC ACID	β -HYDROXY- BUTYRIC ACID
		kgm.								
Serine, 1.29 gms.	1.59	6	I				2.90			
			II	5.92	15.44		2.87			
			III	6.67	24.68		3.70		0.259	
			IV	5.60	17.02		3.03	11.0	0.227	
Cysteine, 14.88 gms.....	1.72	16	I				3.73			
			II	7.83	28.32		3.62		0.217	
			III	10.23	38.12		3.72		0.154	
			IV	8.73	29.73		3.40	8.4	0.136	
			V	8.04	26.54		3.30		0.076	
Cysteine, 15.73 gms.....	1.82	11	I				3.41			
			II	6.11	20.32		3.32		0.053	
			III	7.43	30.36		4.09		0.054	
			IV	7.35	24.90		3.39	12.2	0.080	
Valine, 20 gms.	2.39		I				3.46			
			II	4.04	13.18		3.26		0.123	
			III	5.20	12.14		2.33		0.043	
			IV	4.38	13.86		3.16	1.8	0.253	

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TABLE I—Continued.

SUBSTANCE	NITROGEN AS SUB- STANCE	WEIGHT OF DOSE	PERIOD	TOTAL NITROGEN	GLUCOSE	C/N	EXTRA GLUCOSE	AMINOACID AS SUB- STANCE	AMINOACID AS SUB- STANCE
		10	I			3.27			
			II	4.04	13.60	3.36		0.720	
Valine, 7 gms.	0.84		III	4.62	13.85	3.00	0.5	0.440	
			IV	3.69	11.52	3.12		0.512	
		10	I			3.61			
			II	5.32	17.48	3.29		1.921	
Valine, 9.27 gms	1.10		III	5.48	12.56	2.29	0	1.368	
			IV	4.98	16.80	3.37		0.781	
			V			3.40			
		12	I	3.27	13.02	3.99		0.081	
Isoleucine, 15			II	4.35	14.36	3.30	3.8	0.126	
gms.	1.60		III	3.50	12.01	3.43		0.281	
Isoleucine, 15			IV	3.57	12.25	3.43		0.347	
gms.	1.60		V	4.99	14.48	2.90	2.9	0.306	
		13	I			3.50			
Leucine,			II	5.31	18.38	3.46		0.471	0.735
15 grams.	1.60		III	6.09	21.72	3.25	4.0	0.430	0.530
		13	I			3.21			
Ornithine,			II	3.37	10.78	3.20		0.168	
9.68 grams.	2.06		III	4.95	15.08	3.04	5.5	0.549	
			IV	3.69	11.52	3.12		0.512	
		10	I			3.52			
			II	3.19	10.71	3.36		0.042	
Lysine,			III	6.12	12.99	2.12	0	0.045	
12.69 grams.	2.43		IV	4.83	13.92	2.88		0.063	
		17	I			3.35			
			II	13.04	43.41	3.33		0.140	
Lysine,			III	13.54	38.85	2.87	3.3	0.150	
16.43 grams.	3.15		IV	13.34	43.52	3.26		0.198	
		9	I			2.84			
			II	3.47	10.12	2.91		0.068	
Arginine,			III	6.53	18.54	2.84	10.4	0.278	
15 grams.	4.85		IV	5.37	12.13	2.26		0.110	

TABLE I—Continued.

SUBSTANCE	NITROGEN OF SUBSTANCE	WEIGHT OF DOG	PERIOD	TOTAL NITROGEN	GLUCOSE	G N	EXTRA GLUCOSE	ACETOACETIC ACID	β -HYDROXY- BUTYRIC ACID
		kgm.							
Arginine, 6.89 grams	2.22	7	I	2.99	8.17	2.73	2.4	0.052	
			II	4.83	12.05	2.49		0.187	
			III	3.91	8.05	2.06		0.071	
Arginine, 12.58 grams	4.05	9	I			3.08	8.5		
			II	5.22	14.41	2.76			
			III	8.27	20.80	2.51			
Histidine, 13.2 grams	3.58		I			3.53	4.4		
			II	3.41	11.97	3.51		0.037	
			III	6.36	14.58	2.29		0.135	
			IV	3.57	11.01	3.24		0.044	
			V	3.20	10.14	3.15		0.078	
Histidine, 9.9 grams	3.37	6	I			3.81	4.6		
			II	3.87	14.01	3.62		0.056	
			III	3.92	13.55	3.45		0.008	
			IV	4.85	12.43	2.56		0.009	
			V	4.24	13.50	3.18		0.007	
Histidine, 11.60 grams	3.95	6	I	3.21	10.27	3.20	3.2	0.144	
			II	5.18	11.52	2.22		0.214	
			III	5.14	12.05	2.34		0.141	
Histidine, 11.1 grams	3.01	12	I			3.38	2.6		
			II	6.74	21.02	3.13		0.026	
			III	10.00	25.33	2.53		0.206	
			IV	7.26	21.88	3.02		0.489	

TABLE II.

SUBSTANCE	NITROGEN OF SUBSTANCE	WEIGHT OF DOG	PERIOD	TOTAL NITROGEN	GLUCOSE	C N	EXTRA GLUCOSE	ACETOACETIC ACID	β -HYDROXY- BUTYRIC ACID
		kgm.							
Histidine, 8.38 grams...	2.46	7	I			3.99			
			II	1.45	5.68	3.91		0.016	
			III	2.65	5.76	2.17	0	0.009	
			IV	2.51	4.52	1.80		0.014	
Histidine, 11.18 grams..	3.28	7	I			3.46			
			II	4.24	14.80	3.49		0.260	
			III	6.47	16.72	2.59	2.2	0.127	
			IV	5.07	14.14	2.79		0.315	
Phenylalanine, 11 grams.....	0.93	7	I	3.48	12.67	3.64		0.734	0.97
			II	4.55	15.10	3.32	1.2	2.397	2.52
			III	3.80	12.56	3.30		1.821	1.46
			IV	3.61	12.66	3.51			1.56
Tyrosine, 20 grams.....	1.55	18	I			3.05			
			II	8.69	30.25	3.48		0.140	0.31
			III	9.32	32.16	3.45	0	0.328	0.70
			IV	8.74	27.18	3.11		0.414	0.79
			V	8.93	28.19	3.16		0.153	0.78
Tryptophane, 14.5 grams...	1.99	7	I			3.63			
			II	3.44	13.20	3.80		0.152	
			III	4.52	15.18	3.36	2.7	0.149	
			IV	5.12	15.94	3.11		0.294	
α -Hydroxyiso- valeric Acid, 15 grams.....		12	I			3.41			
			II	7.04	24.93	3.40		0.255	
			III	6.16	19.80	3.21	0.9	1.132	
			IV	7.22	20.72	2.87		0.346	

TABLE III.

SUBSTANCE	WEIGHT OF SUBSTANCE	WEIGHT OF DOG	VOLUME PERFUSION FLUID	TIME OF PERFUSION	ACETO- ACETIC ACID FOUND
	<i>grams</i>	<i>kgm.</i>	<i>cc.</i>	<i>min</i>	<i>mgm.</i>
Blank *		11	1100	50	51
Ornithine Carbonate.....	2.0	12	1100	45	42
Lysine Carbonate.....	2.0	15	1200	45	60
Lysine Carbonate.....	2.0	6	1070	50	67
Arginine Carbonate.....	3.5	13	1000	45	44
Histidine Carbonate*.....	2.5	12	1200	50	86
Proline.....	2.0	9	950	45	49
Di-iodotyrosine.....	2.0	7	1000	50	44
Tryptophane.....	1.5	13	1210	45	54

* Average of many experiments.

A NEW METHOD OF ISOLATING TRYPSIN.

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(Received for publication, February 12, 1913.)

INTRODUCTION.

It has been pointed out by Robertson¹ that "if one drop of a saturated solution of safranin (Grübler) be added to from 5 to 10 cc. of a neutral or very faintly alkaline 0.5 per cent solution of trypsin (Grübler) a light, flocculent, colored precipitate slowly appears on standing and gradually settles." Robertson assumes that this precipitate is a compound of trypsin and safranin and infers that trypsin, in faintly alkaline or neutral solutions, behaves like an acid and combines with the color-base safranin to form an insoluble salt.

The present investigation was undertaken, in the first place, with a view of testing the accuracy of Robertson's assumption that the substance precipitated by safranin is actually trypsin; and, in the second place, the correctness of this assumption having been proven, to endeavor to utilize this precipitate for the isolation of the proteolytic agent from pancreas extracts and commercial preparations of "trypsin."

The proteolytic activity of the safranin precipitate.

I have prepared the safranin precipitate from aqueous solutions of Grübler's and of Fairchild's tryptins and from aqueous extracts of sheep's pancreas and liver, prepared by grinding up the organs with sand and an equal weight of water and filtering. The precipitate was obtained by adding to these solutions three-eighths of their volume of an 0.8 per cent solution of Grübler's safranin.

The yield was always very small. Two and one-half grams of Grübler's trypsin dissolved in 100 cc. of water yielded, on the aver-

¹ T. B. Robertson: This *Journal*, ii, p. 343, 1907.

age, a precipitate weighing from 60 to 75 mgm. when washed in alcohol and dried over sulphuric acid. Owing to its content of safranin the precipitate is of a deep red color and it is, moreover, almost insoluble in water. I have made many attempts to extract the safranin from the compound by repeatedly washing with alcohol. The compound would appear to be slightly soluble in alcohol, however, as the amount of material diminished progressively during this process. It proved, furthermore, impossible to extract the color from it, although the tint changed from red to purple. Prolonged extraction with alcohol also caused the precipitate to become very sticky and rendered it difficult or impossible to scrape it off the filter. I attempted to employ other solvents for extracting the safranin from the precipitate, namely, methyl alcohol, chloroform and benzin, but these were even less effective than ethyl alcohol and ether.

Despite its very slight solubility in water the safranin precipitate from Grüber's trypsin and pancreas extracts exerts a very energetic proteolytic action. The action of the precipitate from Fairchild's trypsin was only slightly inferior.

To 100-cc. samples of a 2 per cent solution of casein in dilute KOH, neutral to phenolphthalein, I added 2 cc. of water containing 20 mgm. of the safranin precipitate from various sources suspended in it. For the purpose of comparison I added to a similar sample of casein solution a solution of 20 mgm. of Grüber's trypsin in 2 cc. of water. After stirring, these mixtures were allowed to stand for three hours at 35°C. I then determined the relative amounts of casein digested, employing Robertson's ² refractometric method and obtained the following results:

TABLE I.

			GRAMS OF CASEIN DIGESTED PER 100 CC. OF DIGEST AFTER 3 HOURS' STANDING AT 35°C.
Safranin precipitate	(Grüber)	1.144
"	"	"	0.934
"	"	(Fairchild)	0.724
"	"	(Pancreas)	1.029
"	"	"	0.934
"	"	(Liver)	0.514
Trypsin	(Grüber)	1.973

² T. B. Robertson: *This Journal*, xii, p. 23, 1912.

It will thus be seen that the proteolytic activity of these preparations was comparable with that of Grüber's trypsin, although they were nearly insoluble, while Grüber's trypsin is nearly completely soluble. We may infer, therefore, that if it were possible to free the preparation from safranin and so obtain it completely in solution its proteolytic activity would be far higher than that of Grüber's trypsin. Hence it is certain that trypsin is actually precipitated by safranin, and probable, also, that this precipitation separates the trypsin from the large proportion of inert impurities present in other preparations.

The presence of a large proportion of relatively inert substances in commercial preparations of trypsin.

If we remove the substance precipitable by safranin by adding three-eighths of its volume of an 0.8 per cent solution of safranin to a 2.5 per cent solution of Grüber's trypsin and filtering, on adding several volumes of alcohol to the filtrate, we obtain a rather heavy, white and flocculent precipitate. On adding to this filtrate progressively increasing volumes of alcohol we obtain progressively increasing yields of precipitate until a maximum yield is obtained by the addition of from five to six volumes of absolute alcohol. This is illustrated by the following results:

TABLE II.

25 cc. filtrate plus	25 cc. absolute alcohol	no precipitate.
" " " " 50 "	" " " "	trace.
" " " " 75 "	" " " "	0.020 grams.
" " " " 100 "	" " " "	0.150 "
" " " " 125 "	" " " "	0.225 "
" " " " 150 "	" " " "	0.255 "
" " " " 175 "	" " " "	0.225 "
" " " " 200 "	" " " "	0.265 "
" " " " 225 "	" " " "	0.265 "

The average yield of the alcohol precipitate was from 0.8 to 1.2 grams per 100 cc. of the filtrate to which 600 cc. of absolute alcohol were added, corresponding to a yield of from 1.1 to 1.6 grams from 2.5 grams of Grüber's trypsin. It is white in color or slightly pink owing to the presence of traces of safranin. It is readily soluble in water, but possesses extremely little proteolytic activity (see table III).

In the filtrate obtained after the removal of the safranin- and alcohol-precipitable substances from Grüber's trypsin colorless needles were deposited upon evaporation. These were probably crystals of magnesium sulphate.

An alcohol precipitate can be obtained by adding alcohol directly to a pancreas extract or to a solution of Grüber's trypsin. This precipitate is white in color and contains the active substance present in the safranin precipitate, as shown by its high proteolytic activity (see table III). Only a relatively small amount of the alcohol precipitate can be obtained from Fairchild's trypsin.

The precipitate which is obtained by adding alcohol directly to a solution of Grüber's trypsin is soluble in water. On adding safranin to this solution a precipitate is obtained.

After the removal of the safranin- and alcohol-precipitable substances from aqueous extract of sheep's pancreas, yet another substance can be precipitated by the addition of ether. This substance is soluble in alcohol and ether, but is insoluble in a mixture of the two in the rather definite proportion of six parts of alcohol to two of ether. It is soluble in mixtures of chloroform and ether and does not yield the biuret reaction. When dry it is white or faintly tinged with pink and slimy in consistency. This substance could not be obtained from Grüber's or Fairchild's trypsin.

The proteolytic activities of the alcohol and ether-alcohol precipitates were determined in a manner described above. In each case 20 mgm. of the substance were dissolved in 2 cc. of distilled water and added to 100 cc. of a 2 per cent solution of casein. The amounts of casein digested were determined after three hours' digestion at 35°C. The results are shown in table III.

From these figures it is clear that as compared with the safranin precipitate or with the "direct" alcohol precipitate the alcohol precipitate which is obtained from Grüber's trypsin or from pancreas extract after removal of the safranin-precipitable substance possesses extremely little proteolytic action, no more, in fact, than might be attributable to contamination with a trace of the safranin-precipitable substance. This fact confirms the view that the substance precipitated by safranin is actually trypsin and also shows that Grüber's and Fairchild's trypsin contain a large proportion of inert substances. It is also clear that trypsin (combined, however, with safranin) can be prepared in a much purer condition,

TABLE III.

GRAMS OF CASEIN DIGESTED
PER 100 CC. OF DIGEST AFTER
3 HOURS' STANDING AT 35°C.

(1) Precipitate obtained by adding alcohol directly to Grüber's trypsin.....	0.618 grams.
(2) Precipitate obtained by adding alcohol directly to pancreas extract.....	1.447 "
(3) Precipitate obtained by adding alcohol to the filtrate after removing the safranin-precipitable substance from Grüber's trypsin.....	0.105 "
(4) Another precipitate of the same	0.211 "
(5) " " " " "	0.092 "
(6) " " " " "	0.198 "
(7) Precipitate obtained by adding alcohol to the filtrate after removing the safranin-precipitable substance from pancreas extract.....	0.105 "
(8) Another precipitate of the same	0.198 "
(9) " " " " "	0.092 "
(10) Ether-alcohol precipitate from pancreas extract (after removal of the safranin- and alcohol-precipitable substances).....	0.000 "
(11) Another precipitate of the same.....	0.105 "

in one operation, namely, by adding safranin to an aqueous pancreas extract and collecting the precipitate.

Each of the fractions isolated, namely, the safranin, alcohol and ether-alcohol precipitates, were tested for lipolytic activity by employing triacetin as a substrate. None of them caused any splitting of this fat.

SUMMARY.

1. The substance, which is precipitated by the addition of safranin to aqueous solutions of Grüber's or Fairchild's trypsin or to aqueous extracts of pancreas, has a strong proteolytic action.

2. This precipitate contains safranin and is very sparingly soluble in water. The author has not yet been able to extract the safranin from the compound or to render it more soluble in water.

3. The safranin-precipitable substance having been removed from aqueous solutions of commercial trypsin or pancreas extracts, considerable quantities of substances remain in solution which are precipitable by alcohol (Grüber's and Fairchild's trypsin and pancreas extract) and by an alcohol-ether mixture of definite composition (pancreas extract). These substances are practically devoid of proteolytic activity.

STUDIES ON THE CONDITIONS AFFECTING THE FORMATION AND EXCRETION OF FORMIC ACID.

THE ESTIMATION OF FORMIC ACID IN URINE.

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But little is known of the excretion of the volatile fatty acids in urine. Of their constant presence there can be no doubt, but knowledge as to their amount and the conditions affecting their formation and excretion is fragmentary. The reason for this is, no doubt, in part due to the inadequacy of existing methods for their determination and separation. For some time, we have been concerned with the problems of volatile fatty acid excretion and at present have occupied ourselves with devising a method for the estimation of the simplest of these acids, namely, formic acid. The fact that formic acid has characteristic reducing properties not shared by the other members of the group, tends to simplify the problem of its determination; but it required but little work to reach the conclusion that an accurate estimation of formic acid in a complex organic fluid, such as urine, is a very difficult matter. It is doubtless for this reason that no trustworthy estimation of formic acid in urines has been, as yet, recorded.

Hitherto, the methods made use of for the isolation of volatile fatty acids in urine have been based upon their preliminary separation by distillation with or without steam from the acidified urines. But at the outset, this process introduces most serious error. The prolonged action of mineral acids upon carbohydrate and other substances, invariably present to a greater or less extent in urines, results in volatile fatty acid production, especially formic acid. In the case of diabetic urines, enormous quantities of formic acid may be produced in this manner, owing to the well-known cleavage of glucose into laevulinic and formic acids. The errors arising from these causes may be diminished by using weaker acids for

liberating the volatile acids, by distilling under reduced pressure at lower temperatures and by avoiding undue concentration of the urine during distillation; but the method, in our hands at least, is fundamentally unsatisfactory.

In connection with attempts to devise a better method, other sources of error were frequently encountered. One of these, if overlooked, is apt to result in gross inaccuracies. Thus, if a urine be rendered alkaline with carbonate of soda and evaporated on the water bath in order to concentrate the urine without loss of volatile fatty acids, it is found that the concentrated urine, especially when carbohydrates are present, may contain several times as much volatile fatty acids after concentration as before. Thus, for example, diabetic urines from dogs which actually contained from 20-50 mgms. of formic acid in the twenty-four hours' collection, after evaporation to one-half the original volume on the water bath with a slight excess of carbonate of soda, were found to contain from 80 to over 1300 mgms. The same change is observed, but to a much lesser extent, in urines free from excess of carbohydrates.

An additional difficulty in arriving at a correct estimate of formic acid in urine was found in the curious variations shown in the formic acid contents of urines on standing. Frequently an increase is observed, but often there is a decrease even when the urine is apparently in good condition. Strong antiseptics are not admissible, but even when the urine is preserved at low temperatures in the presence of toluene, it is important to commence the analysis as soon as possible. Urines showing any bacterial contamination must of course be rejected and, since organisms may act very rapidly, especially in urines containing sugar, it is important that samples should be protected from decomposition as soon as possible after voiding. Even slight fecal contamination at once invalidates the results.

The foregoing considerations led us to the belief that in order to attempt the estimation of formic acid and other volatile acids in urines, it would be necessary to devise a method which would remove the acids from the main bulk of urinary constituents with a minimum of chemical manipulation. With this end in view, we have worked out a procedure which, while far from ideal, does, we believe, offer substantial advantage over current methods.

The method is based upon the observation that it is perfectly feasible, by means of ether, to extract at low temperatures all of the volatile fatty acids from acidified urine or other dilute aqueous solutions.¹ The extraction is performed in any of the usual forms of apparatus arranged for continuous extraction, and in order to prevent the volatile acids, once extracted from the aqueous solution, from returning, we place an excess of carbonate of soda solution in the ether reservoir, thus promptly neutralizing and fixing the volatile acids as fast as they are extracted. By this means the volatile acids are obtained in alkaline solution free from the main bulk of urinary constituents, carbohydrates, purine bodies, amino-acids, etc., which interfere with an accurate estimation of formic or other volatile acids.

On acidifying the sodium carbonate solution with phosphoric acid and distilling in steam, the distillate contains all of the volatile fatty acids and may be used for the determination of formic acid by appropriate methods. The method based upon the reduction of mercuric chloride to calomel has been found by us to be the most satisfactory. The details of the method are described later.

The extraction of formic acid from aqueous solutions by ether.

Preliminary experiments were made to determine the conditions governing the extraction of formic acid by ether. About half a gram of formic acid in 130 cc. of water was extracted with ether in a continuous extractor for varying lengths of time. Excess of alkali was placed in the ether reservoir. In order to determine the amount of formic acid still remaining in the aqueous solution after varying times of extraction, 5 cc. or more of the solution were removed at intervals and titrated against decinormal baryta.

In experiments I and II, the return flow of ether was fairly rapid, though not a continuous stream, but in experiment III, the flow of ether was much slower. The results show that formic acid

¹ The fact that formic acid can be extracted from aqueous solutions with ether, appears not to be generally known. Curtius and Franzen, in order to demonstrate the presence of formaldehyde in plants, oxidized the aldehydes to acids and extracted with ether to remove the higher acids. This procedure must lead to some loss of formic acid. (*Ber. d. deutsch. chem. Gesellsch.*, xliv, p. 1715, 1912.)

EXPERIMENT	TIME OF EXTRACTION	TITRATION OF 5 CC. AQUEOUS SOLUTION WITH $\frac{N}{10}$ BARTTA	PERCENTAGE OF FORMIC ACID EXTRACTED BY ETHER
I	Before extraction	4.20	0
	After 1 hour	1.65	61
	After 2 hours	0.35	92
	After 3 hours	0.05	99
	After 4 hours	Less than 1 drop	100
II	Before extraction	4.20	0
	After 1 hour	1.20	71
	After 2 hours	0.40	90
	After 3 hours	0.15	96
	After 4 hours	Less than 1 drop	100
III	Before extraction	5.10	0
	After 1 hour	3.50	31
	After 3 hours	0.40	92
	After 4 hours	0.15	97
	After 6 hours	Less than 1 drop	100

is comparatively rapidly and completely removed from its aqueous solution when extracted with ether under the existing conditions. After four hours' extraction almost the whole of the formic acid had been removed. In our subsequent determinations of formic acid, we allowed the extraction to continue for twelve hours, thus allowing a considerable margin of safety.

We have found that in order to recover the formic acid after extraction by ether and fixation with alkali, it is most convenient to separate the aqueous portion from the ether, acidify strongly with phosphoric acid and distil in a current of steam. The dilute formic acid solution may then be neutralized with a distinct excess of caustic soda, evaporated on the water bath and is then, after neutralization with acetic acid, ready for gravimetric estimation by means of the mercuric chloride method. That the procedure when carefully carried out does not involve loss was shown by taking 0.0964 gram of formic acid and recovering 0.0963, 0.0980, 0.0968 gram in three consecutive experiments.

Conditions affecting the determination of formic acid by mercuric chloride.

The determination of formic acid, based upon its reducing action upon mercuric chloride with formation of calomel, has been studied by Scala,¹ Lieben,² Leys³ and others. As there appeared to be a certain lack of agreement as to the most favorable conditions, we have reinvestigated some of the principal points.

1. *Time and mode of heating.* The reaction between formic acid and mercuric chloride is a slow one and needs prolonged heating for its completion. Since heavy precipitates of calomel are apt to result in violent "bumping," if the solution be boiled, we prefer to immerse the flasks provided with an air-cooled condenser tube, in a bath of boiling water. The calomel was collected on weighed Gooch crucibles, washed and dried in the water-oven. The subjoined results show that it is necessary to heat for at least six hours

SERIES	TIME OF HEATING	MERCURIC CHLORIDE USED	CALOMEL FORMED	FORMIC ACID PRESENT	FORMIC ACID FOUND	DIFFERENCE
	hours	grams	gram	gram	gram	gram
I	1	6.6	0.9211	0.0957	0.0900	-0.0057
	3	6.6	0.9556	0.0957	0.0933	-0.0014
	4	6.6	0.9719	0.0957	0.0949	-0.0008
	6	6.6	0.9802	0.0957	0.0957	
II	1.5	4.9	0.4041	0.0483	0.0394	-0.0089
	2.5	4.9	0.4481	0.0483	0.0437	-0.0046
	3.5	4.9	0.4721	0.0483	0.0461	-0.0022
	4.5	4.9	0.4808	0.0483	0.0470	-0.0013
	5.5	4.9	0.4863	0.0483	0.0475	-0.0008
	6.5	4.9	0.4935	0.0483	0.0482	-0.0001
III	5	4.9	0.4935	0.0483	0.0482	-0.0001
	6	4.9	0.4936	0.0483	0.0482	-0.0001
	7	4.9	0.4958	0.0483	0.0484	+0.0001
	8	4.9	0.4953	0.0483	0.0484	+0.0001
	9	4.9	0.4955	0.0483	0.0484	+0.0001
	10	4.9	0.4979	0.0483	0.0486	+0.0003

¹Scala: *Gaz. chim. ital.*, xxvi, p. 394, 1890.

²Ad. Lieben: *Monatsh. f. Chem.*, xiv, p. 753, 1893.

³A. Leys: *Bull. de la soc. chim. de Paris*, (3), xix, p. 472, 1898.

2. *Amount of mercuric chloride.* It is well recognized that, in order to obtain accurate results, the presence of a considerable excess of mercuric chloride is necessary. Our experiments (see following table) show that under the conditions of heating chosen by us it is essential to have at least six to eight times the amount theoretically necessary. In practice it is a good plan to use an amount of mercuric chloride equivalent to about one hundred times the weight of formic acid anticipated.

SERIES	TIME OF HEATING	MERCURIC CHLORIDE USED	CALOMEL FORMED	FORMIC ACID PRESENT	FORMIC ACID FOUND	DIFFERENCE
	<i>hours</i>		<i>gram</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>
I	6	2 \times theor. amt.	0.8789	0.0957	0.0858	-0.0099
	6	4 \times theor. amt.	0.9635	0.0957	0.0940	-0.0017
	6	6 \times theor. amt.	0.9802	0.0957	0.0957	
II	6	3 \times theor. amt.	0.4021	0.0477	0.0392	-0.0085
	6	4 \times theor. amt.	0.4316	0.0477	0.0421	-0.0056
	6	5 \times theor. amt.	0.4566	0.0477	0.0446	-0.0031
	6	6 \times theor. amt.	0.4639	0.0477	0.0452	-0.0025
	6	7 \times theor. amt.	0.4810	0.0477	0.0469	-0.0008
	6	8 \times theor. amt.	0.4797	0.0477	0.0468	-0.0009

3. *Effect of sodium acetate and of acetic acid.* Since, in the course of estimating the formic acid in urine, the distillates were made alkaline with caustic soda and then concentrated and it was proposed to acidify with acetic acid before heating with mercuric chloride, it was necessary to investigate the effect of sodium acetate and of acetic acid. The subjoined results show that under the existing conditions an excess of acetic acid up to at least 30 cc. of decinormal strength was without effect. Large amounts of sodium acetate tend to give high results probably due to the precipitation of a little mercurous acetate. Such amounts as occur in the course of urinary analysis would be without effect.

FORMIC ACID TAKEN	$\frac{N}{10}$ ACETIC ACID ADDED	CHYST. SODIUM ACETATE ADDED	CALOMEL FORMED	FORMIC ACID FOUND	DIFFERENCE
<i>gram</i>	<i>cc.</i>	<i>grams</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>
0.0477			0.4846	0.0473	-0.0004
0.0477	10		0.4841	0.0472	-0.0005
0.0477	30		0.4868	0.0475	-0.0002
0.0477	10	2	0.4902	0.0487	+0.0010
0.0477		5	0.5008	0.0488	+0.0011
0.0477		10	0.5107	0.0498	+0.0021

The estimation of formic acid in urine.

The procedure that has been found most satisfactory is as follows: As large a volume of fresh urine as may be conveniently handled is precipitated with solid ammonium sulphate, using about 20 grams for each 100 cc. of urine. Aliquot portions of the filtrate are acidified with phosphoric acid and extracted for twelve hours with a rapid flow of ether. We have commonly used 250 cc. of urine and 10 cc. of 50 per cent phosphoric acid. It is well to use ether previously shaken with caustic soda solution. The flask in which the ether is boiled contains about 20 cc. of 5 per cent sodium carbonate solution.

At the close of the extraction, the contents of the flask are transferred to a separatory funnel and the alkaline solution is allowed to flow into a flask suitable for steam distillation. The ether is washed twice with a little water and the washings added to the flask. The whole is then acidified with phosphoric acid and the volatile acids recovered by distilling in a rapid current of steam. Ordinarily, it suffices to collect a liter of distillate. If necessary, the distillate is filtered through a wet filter paper in order to remove traces of higher fatty acids. It is convenient to determine the total acidity by titrating an aliquot part of the distillate (100 cc.) with decinormal baryta and phenolphthalein. Often considerable variation in the acidity, as determined by titration of duplicates, may occur, owing to the presence of carbon dioxide in varying quantity. The remainder (900 cc.) is then made distinctly alkaline by adding caustic soda solution and concentrated on the water bath to about 50 cc. It is convenient to neutralize with alkali of known strength and to add a definite excess—say 20 cc. of decinormal solution—as in this case the subsequent acidification is most readily managed. Dilute acetic acid is now added, after cooling, in amount slightly more than that necessary to neutralize the excess of caustic soda, so as to render the solution distinctly acid to litmus. After filtering into an Erlenmeyer flask, excess of mercuric chloride is added. For each cubic centimeter of total acidity measured with decinormal alkali, it is well to use 5 cubic centimeters of saturated mercuric chloride solution. The addition of the mercuric chloride is occasionally followed by an immediate trifling turbidity which cannot be easily removed by filtra-

tion. The origin of this turbidity is unknown but it is not due to lower fatty acids, lactic, benzoic, phenylacetic or hydroxyphenyl-acetic acids, and since the relative weight of calomel obtained from formic acid is so large, it does not introduce a significant error.

The flask is now provided with a short air-cooled condenser tube and heated for at least six hours by immersion in a boiling water bath. After cooling, the precipitate is filtered off on a weighed Gooch crucible and washed with 100 cubic centimeters of cold 5 per cent hydrochloric acid, then successively with water, alcohol and ether. Finally it is dried for about two hours in the water-oven and weighed (1 gram calomel = 0.0977 gram formic acid). The object of washing with the hydrochloric acid is to remove some slight impurities which are apt to occur in the precipitates obtained from urines, while the calomel is substantially unattacked, provided the hydrochloric acid is not used hot. It is advisable to make blank tests on the reagents as they will usually be found to give 5-10 mgms. of precipitate equivalent to 0.5-1 mgm. of formic acid.

Oxalic, lactic and crotonic acids do not act as disturbing factors in this estimation of formic acid.

Formic acid as a product of intermediary metabolism.

The constant presence of formic acid in urines and the comparative ease with which it undergoes oxidation in the animal body, makes it appear probable that it is an important product of intermediary metabolism; but there is an almost entire lack of information concerning the origin of the acid.

Pohl showed that an increased formic acid excretion followed administration of formaldehyde, methylamine, methyl alcohol and some derivatives of these substances; but these results have little bearing on the processes of normal metabolism. More recently Steppuhn and Schellbach⁵ reported an increase in formic acid output following glucose administration by mouth and they believe that its formation may be demonstrated when liver tissue undergoes autolysis in the presence of glucose. The risk of bacterial decomposition of sugar with production of formic acid in these experiments would appear to be great and in addition we are inclined to believe that the method of analysis employed was not adequate to

⁵ *Zeitschr. f. physiol. Chem.*, lxxx, p. 274, 1912.

the difficult task of estimating formic acid in the presence of much glucose.

Our experiments are concerned with the following points:

I. Formic acid in normal human urines.

II. The influence of starvation, and of carbohydrates and proteins on the formic acid excretion.

III. The relation of higher fatty acids and other substances to formic acid production.

I. *Formic acid in normal urines.* Twelve analyses of normal human urines, from individuals on mixed diets, gave results varying from 29.9 to 118.6 mgms. in the twenty-four hours with an average excretion of 60.3. There was no obvious relation between weight, urine volume or nitrogen excretion. These data are therefore omitted.

On increasing the consumption of carbohydrates, the formic acid excretion shows a tendency to increase (35.0–175 mgms. per 24 hours. Average, 96 mgms.). The effect of excessive protein is similar but less marked (34.6–99 mgms. per 24 hours. Average, 67 mgms.). These effects are seen more clearly in the experiments on dogs.

II. *Effect of starvation, carbohydrates and proteins.* The excretion of formic acid in animals rapidly falls on starvation to about one-third the normal amount. The average result of six experiments on dogs weighing 10–12 kgms. is 8.9 mgms. per 24 hours. On administering carbohydrates (potatoes) to these animals a marked increase in formic acid is observed. The average excretion on a potato diet was 51 mgms. On feeding meat freely, a very definite increase over the output during starvation is observed, but the effect is somewhat less than that observed with carbohydrates. The average output on a meat diet for the same animals was 27 mgms.

Since it is known that many intestinal microorganisms may not only ferment but also produce formic acid from both carbohydrates and proteins, it was conceivable that these increases in formic acid excretions were due to bacterial activity. That this explanation does not account for the rise was shown by the fact that a marked increase in formic acid output was found to follow intravenous injections of glucose given to fasting dogs. It seems likely therefore that formic acid is an intermediate product of the catabolism

of carbohydrates and proteins. The relatively small amount of the acid actually excreted is due in part, no doubt, to the fact that it undergoes further oxidation in the body with ease. It is at present impossible to form an estimate of the amount actually produced.

An attempt was made to gain some insight into the protein constituents which were capable of yielding formic acid by administering to cats and rabbits certain amino-acids and allied substances (table III). Glycine, glycollic acid, alanine, lactic acid and aspartic acid led to no definite rise in formic acid excretion, but two experiments, in which relatively large amounts of histidine hydrochloride were given to cats, resulted in a very definite increase in formic acid output. It is noteworthy, too, that phlorhizin glycosuria induced in starving dogs, accompanied by a large increase in protein catabolism, is followed by a rise in formic acid output.

III. *The relation of formic acid to higher fatty acids.* In a preliminary note^a published by two of us (H.D.D. and A.J.W.) it was stated that "the administration of the sodium salts of fatty acids may result in the excretion of from ten to thirty times the normal amount of formic acid." This statement was made on the basis of more than fifteen separate experiments, but as the result of a much larger number, carried out subsequently, we are forced to the conclusion that we largely overestimated the increase and we therefore wish to correct our earlier statement. The later experiments show that while an increase in formic acid output does commonly follow administration of the sodium salts of fatty acids, it seldom amounts to more than three to four times the normal output. In our earlier experiments we were not aware of many of the sources of error in formic acid analyses referred to at the commencement of this paper.

A few of our later experiments were recorded in table III. In order to further test the possibility of formic acid arising from the oxidation of higher fatty acids in the body, experiments were made to determine the formic acid in blood to which sodium acetate and propionate had been added before and after perfusion of the surviving liver of dogs. Our experiments gave negative results and therefore will not be described in detail.

^a This *Journal*, ix, p. 329, 1911.

In conclusion, reference may be made to the possible production of formic acid from caffeine and theobromine when these substances undergo demethylation in the animal body. Preliminary experiments have given encouraging results and the question will be submitted to a careful examination.

SUMMARY.

An improved method for the estimation of formic acid is described.

The formic acid present in the urine is in part of endogenous origin. The excretion of formic acid is greatly reduced during starvation but is largely increased when carbohydrates are given, either by mouth or when glucose is given intravenously. Protein feeding is followed by a similar but smaller increase in formic acid output. Formic acid appears to be a product of the intermediary metabolism of carbohydrates and proteins.

The effect of a number of other substances on formic acid excretion was investigated, including amino, hydroxy and saturated fatty acids.

TABLE I.¹

Effect of fasting, carbohydrates and proteins on formic acid excretion.

EXPERIMENT	WEIGHT OF DOG	FORMIC ACID	TOTAL NITROGEN	DIET	
	<i>kgm.</i>	<i>mgm.</i>	<i>grams</i>		
I	11	6.6	2.5	Fasting	Average for 3 days.
		60.0	4.7	Potatoes and gravy	Average for 2 days.
		27.5	20.5	Meat	Average for 2 days.
II	13	8.3	3.9	Fasting	Average for 3 days.
		30.0	31.5	Meat	Average for 4 days.
		62.0	3.6	Potatoes	Average for 4 days.
		44.0	6.7	Fasting	Phlorhizin glycosuria.
III	12.7	9.5	3.0	Fasting	Average for 2 days.
		32.0	2.1	Potatoes	

¹ All figures refer to twenty-four-hour periods.

TABLE II.

Effect of intravenous injections of glucose on formic acid excretion.

EXPERIMENT	WEIGHT OF DOG	FORMIC ACID	TOTAL NITROGEN	CONDITIONS
	<i>kgm.</i>	<i>mgm.</i>	<i>grams</i>	
I	.11	14.1	4.5	Average for 2 days after 3 days fasting.
		69.0	5.3	200 grams glucose given intravenously. 97.6 grams excreted unchanged.
II	12.7	9.5	3.0	Average for 2 days.
		23.4	3.5	80 grams glucose given intravenously. 33 grams excreted unchanged.
III	8.9	5.7	1.9	Fasting.
		45.8	3.0	71 grams glucose given intravenously. 28.5 grams excreted unchanged.
IV	10	8.2	2.6	Fasting.
		47.8	3.0	100 grams glucose given intravenously. 37 grams excreted unchanged.

TABLE III.

Effect of various substances on formic acid excretion.

ANIMAL	WEIGHT	FORMIC ACID 24 HOURS	TOTAL NITROGEN	SUBSTANCES GIVEN*
	<i>kgm.</i>	<i>mgm.</i>	<i>grams</i>	
{ Cats.....	2-3.5	2.0-5.6		Fasting. Average of 5 experiments.
{ Cats.....	2-3.5	6.3-9.4		Meat diet. Average of 3 experiments.
Cat.....		5.9	1.88	Sodium chloride 100 cc. 0.85 per cent intravenously.
Cats.....	2-3.5	2.1-7.9		Sodium bicarbonate 3-5 grams intravenously. Average of 5 experiments.
{ Rabbit.....	1.9	3.4	1.7	
{ Rabbit.....	1.9	9.1	0.8	Acetic acid, 2 grams by mouth.

* All acids in form of sodium salts.

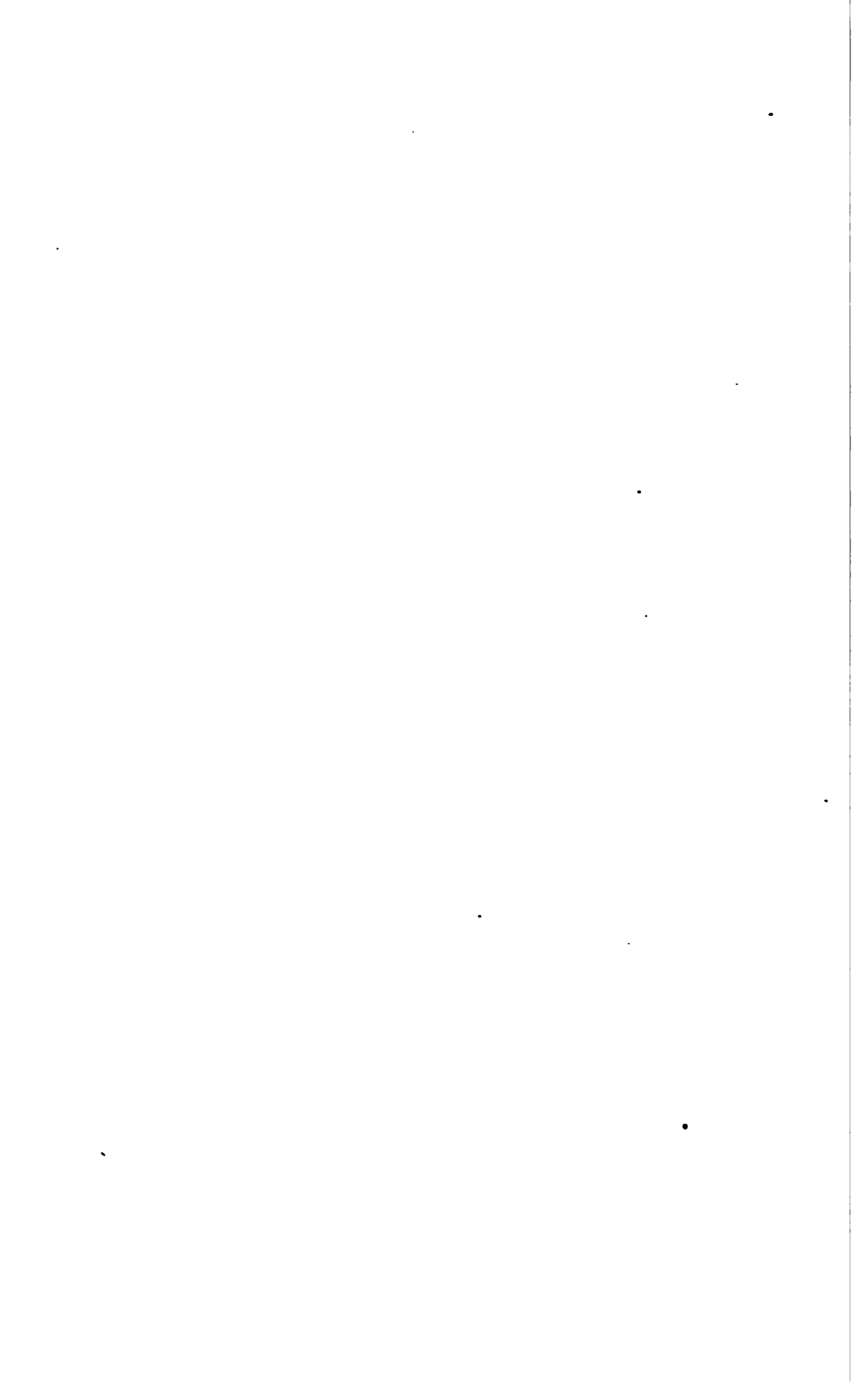
Intravenous injections usually measured 80-150 cc. and lasted one to four hours.

TABLE III—Continued.

ANIMAL	WEIGHT	FORMIC ACID 24 HOURS	TOTAL NITROGEN	SUBSTANCES GIVEN*
	kgm.	mgm.	grams	
Cats.....	2.5-3.0	13.0-44.5		Acetic acid, 3-4.5 grams intravenously.
Cat.....	3.0	23.0	2.34	Propionic acid, 5 grams intravenously.
Cat.....	3.0	19.0		Propionic acid, 7 grams intravenously.
{ Rabbit.....	1.2	5.2	0.8	
{ Rabbit.....	1.2	10.1	1.3	Butyric acid, 2 grams by mouth.
Cat.....	3.5	29.8	4.50	Butyric acid, 5 grams intravenously.
Cat.....		13.7	0.59	Glycollic acid, 4 grams intravenously.
{ Rabbit.....	1.8	7.1	1.9	
{ Rabbit.....	1.8	7.9	1.5	Lactic acid, 2.5 grams by mouth.
{ Rabbit.....	1.2	3.4	1.7	
{ Rabbit.....	1.2	3.7	1.1	Acetoacetic acid, 2.5 grams by mouth.
Cat.....	3.5	11.7	5.22	Glycocoll, 5 grams intravenously.
Cat.....	2.7	9.3	4.5	Alanine, 6 grams intravenously.
Cat.....	3.0	12.3	5.04	Aspartic acid, 6 grams intravenously.
Cat.....	2.8	27.0	2.73	Histidine carbonate, 2 grams intravenously.
{ Cat.....	2.8	6.5		
{ Cat.....	2.8	42.4		Histidine carbonate, 3 grams intravenously.
Cat.....	3.5	82.0		Histidine hydrochloride, 3.5 grams subcutaneously.
Cat.....	3.1	430.0	3.03	Methyl alcohol, 10 grams intravenously.
Cat.....	3.1	8.7	1.89	Ethyl alcohol, 20 grams intravenously.

* All acids in form of sodium salts.

Intravenous injections usually measured 80-150 cc. and lasted one to four hours.



THE RELATIVE INFLUENCE OF WEAK AND STRONG BASES UPON THE RATE OF OXIDATIONS IN THE UNFERTILIZED EGG OF THE SEA URCHIN.

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(Received for publication, March 15, 1913.)

1. About six years ago it was shown by Loeb that bases (NaOH, KOH) can induce artificial parthenogenesis in the eggs of sea urchins and annelids.¹ This action of the bases was suppressed or retarded when the oxidations in the egg were suppressed or retarded by the withdrawal of oxygen from the alkaline solution or by the addition of a small amount of KCN.² He therefore concluded that the bases induced artificial parthenogenesis through an acceleration of the rate of oxidations in the egg. Last summer the same author showed that the weak base NH_4OH is much more efficient for the causation of artificial parthenogenesis than the strong bases NaOH, KOH or tetraethylammoniumhydroxide.³ This he explained on the basis of the fact found by O. Warburg,⁴ and extended by Harvey,⁵ that the weak bases diffuse more readily into the egg while the strong bases do not. This behavior was an analogue to the fact found by Loeb in 1905 that weak acids like CO_2 or the monobasic fatty acids induce membrane formation and development readily in the unfertilized egg of the sea urchin, while the strong acids like HCl or oxalic acid are very ineffective.⁶ This fact he explained on the assumption that the weak acids diffuse readily into the egg while the strong acids do not. While the bases

¹ Loeb: *Pflüger's Archiv*, cxviii, p. 572, 1907.

² Loeb: *ibid.*, cxviii, p. 30, 1907.

³ Loeb: *Journ. of Exp. Zoology*, xiii, p. 577, 1912.

⁴ Warburg: *Zeitschr. f. physiol. Chem.*, lxvi, p. 305, 1910.

⁵ Harvey: *Journ. of Exp. Zoology*, x, p. 507, 1911.

⁶ Loeb: *Univ. of Calif. Publ. Physiol.*, ii, p. 113, 1905; *Biochem. Zeitschr.*, xv, p. 254, 1909.

only acted in the presence of oxygen, the action of the acids was independent of oxidations in the egg.

2. More recently Loeb extended his investigations on the relative efficiency of weak and strong bases for artificial parthenogenesis to a larger number of bases. The egg used was that of an annelid, *Polynoe*. It was found that the bases, in regard to their efficiency for this purpose, may be divided into three groups. The most efficient were the amines of which benzyl, butyl, ethyl and methylamine were tried. Benzyl and butylamine were possibly a little more efficient than ethyl and methylamine. Next in efficiency were NH_4OH and trimethylamine. The least efficient were the strong bases NaOH and tetraethylammoniumhydroxide.

The relative efficiency of these bases for the causation of artificial parthenogenesis was tested in this way that the unfertilized eggs of *Polynoe* were put into solutions containing the same molecular concentration of these various bases. The reciprocal value of the time required for the various bases to cause development was the measure of their relative efficiency. The simple amines acted most quickly, then followed NH_4OH and trimethylamine; the strong bases required more time than either of the two other groups of bases.

3. These experiences suggested an investigation of the influence of the various bases upon the rate of oxidations in the unfertilized egg, to find out whether the weaker bases raised the rate of oxidations more than the stronger bases.

The experiments were carried out on the unfertilized egg of *Strongylocentrotus purpuratus* in Pacific Grove, California. The oxygen consumption was determined according to Winkler's methods. The experiments were made in this way that the oxygen consumption for the same lot of eggs was first determined in a neutral solution and then for the same length of time and the same temperature in an alkaline solution. The experiments were made in a half gram molecular mixture of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ in that proportion in which these three salts are contained in the sea water. The reader who is interested in the details of the method may be referred to our former publications or to those of Warburg.⁷

⁷ Loeb and Wasteneys: *Biochem. Zeitschr.*, xxviii, p. 340, 1910; Warburg: *loc. cit.*

We first give the results of a series of experiments in which the relative influence of various bases was compared. The time of exposure was one hour and twenty-five minutes; the temperature, 3°C. The concentration of the bases chosen was that found most effective in Loeb's previous experiments on artificial parthenogenesis. The oxygen consumption was first measured in a neutral solution and then for the same eggs in the alkaline solution in which 0.3 cc. of $\frac{N}{10}$ of the various bases was added to 50 cc. of the solution.

TABLE I.

NUMBER OF EXPERIMENT	NATURE OF SOLUTION	OXYGEN CONSUMED	ACCELERATION OF RATE OF OXIDATION BY THE BASE
		mgm.	
I	{ Neutral.....	0.28	
	{ 50 cc. neutral + 0.3 cc. $\frac{N}{10}$ NaOH.....	0.40	1.43
II	{ Neutral.....	0.15	
	{ 50 cc. neutral + 0.3 cc. $\frac{N}{10}$ tetra- ethylammoniumhydroxide.....	0.22	1.50
III	{ Neutral.....	0.30	
	{ 50 cc. neutral + 0.3 cc. $\frac{N}{10}$ NH ₄ OH.....	0.81	2.70
IV	{ Neutral.....	0.40	
	{ 50 cc. neutral + 0.3 cc. $\frac{N}{10}$ trimethyl- amine.....	1.19	3.00
V	{ Neutral.....	0.25	
	{ 50 cc. neutral + 0.3 cc. $\frac{N}{10}$ methyl- amine.....	1.18	4.70
VI	{ Neutral.....	0.28	
	{ 50 cc. neutral + 0.3 cc. $\frac{N}{10}$ ethylamine.....	1.35	4.80
VII	{ Neutral.....	0.32	
	{ 50 cc. neutral + 0.3 cc. $\frac{N}{10}$ butyl- amine.....	1.23	3.80
VIII	{ Neutral.....	0.22	
	{ 50 cc. neutral + 0.3 cc. $\frac{N}{10}$ benzyl- amine.....	1.30	5.90

These experiments, which were repeated with the same result, show clearly that the relative efficiency of the bases for inducing artificial parthenogenesis in the unfertilized eggs of *Polynoe* and

the sea urchin runs parallel with their accelerating influence upon the rate of oxidations in the unfertilized egg of the sea urchin. Incidentally it may be stated that NaHCO_3 does not accelerate the rate of oxidations in the unfertilized egg nor does it cause artificial parthenogenesis.

4. We compared next the relative effect of various concentrations of NaOH and NH_4OH upon the rate of oxidations in the unfertilized sea urchin egg, during one hour. We will state only the coefficient of the rate of oxidation in the various solutions, calling the rate in the neutral solution 1.

TABLE II.

AMOUNT OF BASE ADDED TO 50 cc. $\frac{N}{10}$ ($\text{NaCl} + \text{KCl} + \text{CaCl}_2$)	COEFFICIENT OF ACCELERATION OF OXIDATIONS IN	
	NaOH	NH_4OH
0.2 cc. $\frac{N}{10}$	1.35	1.29
0.5 cc. $\frac{N}{10}$	1.87	5.00
0.8 cc. $\frac{N}{10}$	1.74	5.94
1.0 cc. $\frac{N}{10}$	2.06	6.10
1.4 cc. $\frac{N}{10}$	2.32	6.40
2.0 cc. $\frac{N}{10}$	3.42	6.23
2.5 cc. $\frac{N}{10}$	4.57	5.70
3.0 cc. $\frac{N}{10}$	7.63	6.00

The reader will notice the striking difference in the behavior of NH_4OH and NaOH . Very low concentrations of NH_4OH (0.5 cc. per 50 cc. of solution) raise the rate of oxidations in the fertilized egg almost to the maximal height, and a further rise in the concentration has only a slight effect upon the rate of oxidation. Low concentrations of NaOH raise the rate of oxidation only little and its efficiency rises steadily with an increase in its concentration. We could not go beyond the concentrations used in this experiment since the addition of 3 cc. of $\frac{N}{10}$ NaOH to 50 cc. of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ leads already to a cytolysis of the eggs.

It is also of interest to point out that in the eggs of *Strongylocentrotus purpuratus* fertilization by sperm raises the rate of oxidation to about six times that in the unfertilized eggs. This seems to indicate that with NH_4OH it is not possible to raise the rate of oxidations in the unfertilized egg beyond the limit to which it can be raised by the fertilization with sperm. It is not possible to decide whether the same holds true for NaOH .

The fact that NH_4OH reaches its maximum effect at so low a concentration is not confined to NH_4OH but is also shared by the amines, as the following table shows. NH_4OH and ethylamine were compared.

TABLE III.

AMOUNT OF BASE ADDED TO 50 cc. $\frac{N}{10}$ ($\text{NaCl} + \text{KCl} + \text{CaCl}_2$)	COEFFICIENT OF ACCELERATION OF OXIDATIONS IN	
	NH_4OH	Ethylamine
0.1 cc. $\frac{N}{10}$	1.9	1.4
0.2 cc. $\frac{N}{10}$	2.9	3.0
0.4 cc. $\frac{N}{10}$	3.4	4.3
0.8 cc. $\frac{N}{10}$	3.9	4.2

Ethylamine reaches its maximal efficiency at the concentration of 0.4 cc. of $\frac{N}{10}$ base to 50 cc. of the neutral liquid; and for NH_4OH the limit is nearly at the same point as in our previous experiment.

5. It seems natural to connect this difference in the behavior of NaOH and NH_4OH with the difference in the rate of their diffusion into the unfertilized egg. If the rate of diffusion of NaOH is extremely slow and that of NH_4OH fast, it is natural that the maximal rate of oxidation should be reached with a lower concentration of NH_4OH than of NaOH . We determined the consumption of oxygen for the same lot of eggs for eight consecutive hours in 50 cc. of sea water + 1.0 cc. of $\frac{N}{10}$ NaOH . The following table gives the result.

TABLE IV.

Consumption of oxygen at 18° in 50 cc. of normal sea water + 1.0 cc. of $\frac{N}{10}$ NaOH in eight consecutive hours.

	OXYGEN CONSUMED	COEFFICIENT OF OXIDATION
	mgm.	
1st hour.....	0.24	1.00
2d hour.....	0.38	1.57
3d hour.....	0.45	1.87
4th hour.....	0.50	2.08
5th hour.....	0.58	2.42
6th hour.....	0.72	3.00
7th hour.....	0.92	3.84
8th hour.....	0.95	3.96

This table shows that the longer the NaOH acts upon the egg the higher the amount of oxygen becomes which is consumed per

hour. This would agree with the assumption that the NaOH diffuses slowly into the egg and that the increase in the rate of oxidations in the unfertilized egg is determined by the amount of base which has diffused into the egg.

6. It was to be expected that since NH_4OH is very soluble in the egg, *i.e.*, diffuses rapidly into the egg, its maximum effect would be reached during the first hour. This was found to be true, as the following table shows.

TABLE V.

Consumption of oxygen by unfertilized eggs at 18° in 50 cc. of normal sea water + 0.8 cc. of $\frac{N}{10}$ NH_4OH .

		OXYGEN CONSUMED	COEFFICIENT OF OXIDATION
		<i>mgm.</i>	
Normal sea water.....		0.15	1.0
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ NH_4OH	1st hour	0.99	6.7
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ NH_4OH	2d hour	1.03	6.9
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ NH_4OH	3d hour	0.87	5.8
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ NH_4OH	4th hour	0.86	5.7
50 cc. sea water + 0.8 cc. $\frac{N}{10}$ NH_4OH	5th hour	0.83	5.5

7. We intend to investigate in future experiments whether these effects of bases upon the rate of oxidations in the unfertilized eggs are irreversible, *i.e.*, will continue if the eggs are put into normal sea water after the treatment with alkali. But, we have an experiment which possibly serves the same purpose. We measured the amount of oxygen consumed in one hour by the eggs mentioned in the last table in the same solution sixteen and twenty-four hours, respectively, after the experiment. In the meantime the eggs had been kept at a low temperature in normal sea water. The rate of oxidation after sixteen or twenty-four hours was practically the same as in the second hour.

8. These experiments prove two facts, first, that the weaker bases increase the rate of oxidations in the unfertilized egg more than the stronger bases; and second, that this difference is due to the fact that the weaker bases diffuse more rapidly into the egg than the strong bases.

The connection between the oxidative action of bases and artificial parthenogenesis lies in the fact that the essential factor in

artificial parthenogenesis is an alteration of the surface or cortical layer of the egg which results in a membrane formation. Loeb has shown in former experiments that bases cause the swelling and liquefaction of the gelatinous mass (the so-called chorion) which surrounds the immature egg of a mollusc, *Lottia*, and that this action of bases is inhibited by lack of oxygen and by the addition of KCN.⁸ This year the same author convinced himself that weak bases like the amines and NH_4OH bring about the dissolution of the chorion much more rapidly than the strong bases NaOH and tetraethylammoniumhydroxide. It is possible that the induction of artificial parthenogenesis in the sea urchin egg by bases depends upon the occurrence of a similar process in the cortical layer of this egg.⁹ We may imagine that they act by accelerating the rate of oxidation of a substance (existing in the cortical layer of the egg?) whereby the membrane formation and consequently the development of the egg is induced.

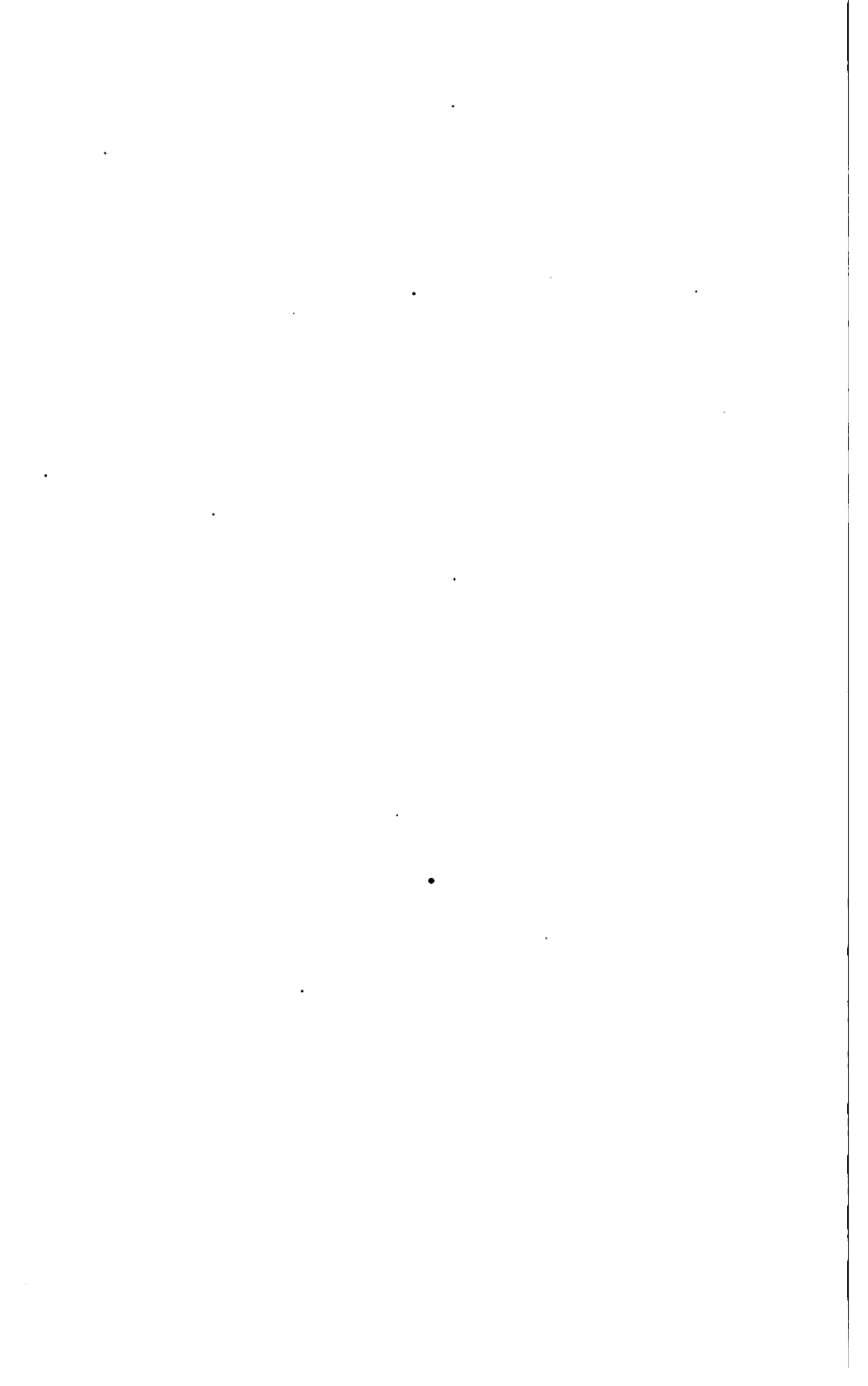
SUMMARY.

The paper shows that the weak bases which are more efficient in causing artificial parthenogenesis are also more efficient in raising the rate of oxidations in the unfertilized egg. This lends further support to the view expressed by Loeb that the bases cause artificial parthenogenesis through an acceleration of the rate of oxidations.

The experiments were carried on at the Herzstein Laboratory in Monterey, California, and the authors express their thanks to Drs. Robertson, Maxwell and Moore for their kind hospitality.

⁸ Loeb: *Univ. of Calif. Publ. Physiol.*, iii, p. 1, 1905.

⁹ Loeb: *Die chemische Entwicklungserregung des tierischen Eies*, Berlin, 1909, p. 181.



FURTHER METABOLISM EXPERIMENTS UPON PARATHYROIDECTOMIZED DOGS.

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(Received for publication, March 19, 1912.)

In a previous paper,¹ the author described a series of metabolism experiments upon parathyroidectomized dogs. The most striking change in the metabolism observed after removal of the parathyroids was the very marked diminution in the excretion of phosphorus in the urine. This was not accompanied by an increase in the elimination of phosphorus in the feces. Apparently there was a retention of phosphorus in the body.

One of the questions that then arose was: Is this retention of phosphorus a primary metabolic disturbance or is it secondary to a retention of sodium or of potassium or of both? The experiments here reported were designed to answer this question. The results leave no doubt that the retention of phosphorus is primary. In none of the experiments was there any indication of a retention of sodium or of potassium preceding that of phosphorus. In three, the fall in the phosphorus elimination occurred a day before the excretion of sodium and potassium was affected. In the other two, the excretion of all three substances was diminished on the day of the operation.

The significance of these experiments is not altogether clear. It may be that, after parathyroidectomy, the ratio between the concentrations of calcium and of sodium and potassium in the body-fluids is altered not so much by loss of calcium as by retention of sodium and potassium. A few analyses of serum indicate that this is the case. Whether or not such a change can be regarded

¹Greenwald: *Amer. Journ. of Physiol.*, xxviii, p. 103, 1911.

as the cause of tetany is, of course, questionable. However, since these changes in the excretion of phosphorus, sodium and potassium are so marked, it seemed desirable that the content of these substances in the tissues be investigated. Some work has already been done on the amount and nature of the phosphorus compounds of the blood and serum of normal and parathyroidectomized dogs. An account of these experiments is given in the following paper.

As in the previous experiments, the author is indebted to Dr. W. G. MacCallum, who was kind enough to operate upon the animals required.

EXPERIMENTAL.

The conduct of the experiments was similar to those previously reported.² The analytical procedures were the same with the following additions and changes:

Phosphates. Titration with uranium acetate, using cochineal as indicator.
Mineral acidity. The Folin method.³

Potassium and sodium. Fifty cc. of urine are ashed, with the aid of a little sulphuric acid, in a platinum dish. The residue is dissolved in water and treated with an excess of barium hydroxide. The precipitate is filtered out and washed thoroughly. A slight excess of sulphuric acid is added to the filtrate. After filtering from the barium sulphate, the liquid is evaporated to dryness in a weighed platinum dish, which is then ignited and weighed. (The trace of calcium present is included but may be disregarded.) The residue is dissolved in about 20 cc. of water, 2 cc. of glacial acetic acid are added and the potassium precipitated as the cobaltinitrite by adding 20 cc. of the solution of Adie and Wood,⁴ drop by drop (this was found to be necessary to ensure correct results). After standing over night the precipitate is filtered off on a Gooch crucible fitted with a piece of filter paper, and washed with 10 per cent acetic acid, as recommended by Thompson and Morgan,⁵ whose directions are followed from this point. The crucible with the precipitate is placed in the beaker in which the precipitation has been made, an excess of barium hydroxide solution is added and the liquid is boiled for two or three minutes. The precipitate of cobalt hydroxide is filtered off and washed. The filtrate and washings are diluted to a definite volume, generally 250 cc. This liquid is then run into a hot, dilute standard

² Greenwald: *loc. cit.*

³ Folin: *Amer. Journ. of Physiol.*, ix, p. 265, 1903.

⁴ Adie and Wood: *Journ. of the Chem. Soc.*, lxxvii, p. 1076, 1900.

⁵ Thompson and Morgan: *Journ. of Ind. and Eng. Chem.*, iii, p. 398, 1911.

olution of potassium permanganate, containing sulphuric acid, until this is decolorized. From the volume required, the amount of potassium is calculated and from this and the weight of the mixed sulphates, the amount of sodium.

TABLE I.

Excretion of phosphorus after incomplete (?) parathyroidectomy. Dog #05.

DATE 1912	WEIGHT	VOLUME	SP. GR.	PHOS- PHATES	REMARKS
<i>February</i>	<i>kilos</i>	<i>cc.</i>		<i>mgm. P.</i>	
6	11.67	385		361	
7	11.72	360	1.019	366	
8	11.70	380	1.020	393	
9	11.70	400	1.019	406	
10	11.70	450	1.018	511	
11	11.70	400	1.020	438	
12		415	1.021	467	Parathyroidectomy, 12 noon, Feb. 11.
13	11.70	400	1.017	353	
14	11.72	330	1.022	324	
15	11.72	380	1.020	445	

At no time did the dog show any evidence of a parathyroid insufficiency. The excretion of phosphorus remained almost unchanged.

TABLE II.

Urinary data. Dog #09.

DATE 1912	VOLUME	SP. GR.	NITROGEN	CHLORINE	PHOS- PHATES	POTASSIUM	SODIUM
<i>February</i>	<i>cc.</i>		<i>grams</i>	<i>mgm.</i>	<i>mgm. P.</i>	<i>mgm.</i>	<i>mgm.</i>
27	600	1.016	7.567	605	651	1027	370
28	460	1.020	7.741	240	562	727	32
29	400	1.018	6.760	173	493	697	28
<i>March</i>							
1	430	1.017	6.578	163	437	627	54
2	200	1.031	5.675	29	11	618	8
3	420	1.014	5.858	102	14	278	28

The dog weighed 13.50 kilos. Parathyroidectomy was performed at 10.30 a.m., March 1. A slight tremor was observed at 2.30 p.m., March 2. This gradually increased in severity until 10.20 a.m., March 3, when the dog was exsanguinated. In spite of the very marked diminution in the excretion of phosphorus on the day of the operation, the elimination of potassium remained unchanged until the following day.

TABLE III.
Urinary data. Dog 212.

DATE 1912	WEIGHT	VOLUME	SP. GR.	NITROGEN	PHOSPHATES	MINERAL ACIDITY
<i>June</i>	<i>kilos</i>	<i>cc.</i>		<i>grams</i>	<i>mgm. P.</i>	<i>cc. $\frac{N}{15}$ acid</i>
13	13.55	540	1.026	12.65	746	440
14	13.50	550	1.022	11.38	718	624
15	13.50	490	1.021	9.56	616	595
16	13.50	400	1.024	9.51	639	619
17	13.60	460	1.026	9.40	551	561
18		140*	1.036	4.80	115	127
18 p.m.		360	1.021	8.34	148	317

* A small quantity of urine was lost.

The parathyroids were removed at 11.30 a.m., June 17. Symptoms appeared on the afternoon of the following day. The dog was bled to death at 4 p.m. On the day of the operation the lessened phosphorus excretion was paralleled by diminished mineral acidity. The latter was increased somewhat on the following day, due, presumably, to a retention of potassium and sodium.

TABLE IV.
Urinary data. Dog 214.

DATE 1912	VOLUME	SP. GR.	NITROGEN	PHOSPHATES	CHLORINE	MINERAL ACIDITY
<i>July</i>	<i>cc.</i>		<i>grams</i>	<i>mgm. P.</i>	<i>mgm.</i>	<i>cc. $\frac{N}{15}$ acid</i>
9	300	1020	6.566	369		
10	360	1020	8.335	511		
11	320	1020	8.170	476		
12			4.960	317		
	430	1026				
13			4.960	317		
14	550	1013	8.880	531	256	435
15	430	1015	7.163	454	241	365
16	350	1020	7.152	425	210	333
17	300	1028	7.290	435	471	96
18	400	1016	8.145	317	162	359
19	350	1021	8.046	348	249	390
20	230	1027	10.000	481	273	453
21	560		8.108	257	245	221
22	450	1016	8.106	147	147	251

The dog weighed 12.40 kilos. Parathyroidectomy was performed at 11 a.m., July 16. There was a slight fall in the excretion of phosphorus but no other indication of parathyroid insufficiency. On July 20, at 4.30 p.m., both thyroids were removed. The output of phosphorus immediately decreased. Slight twitching was noted at 11.30 p.m., July 21. The next

morning, the twitching was more marked and the dog was killed. The very marked drop in the mineral acidity on the day of the first operation cannot, at present, be satisfactorily explained. After the second operation, the mineral acidity fell with the excretion of phosphorus.

TABLE V.
Urinary data. Dog 215.

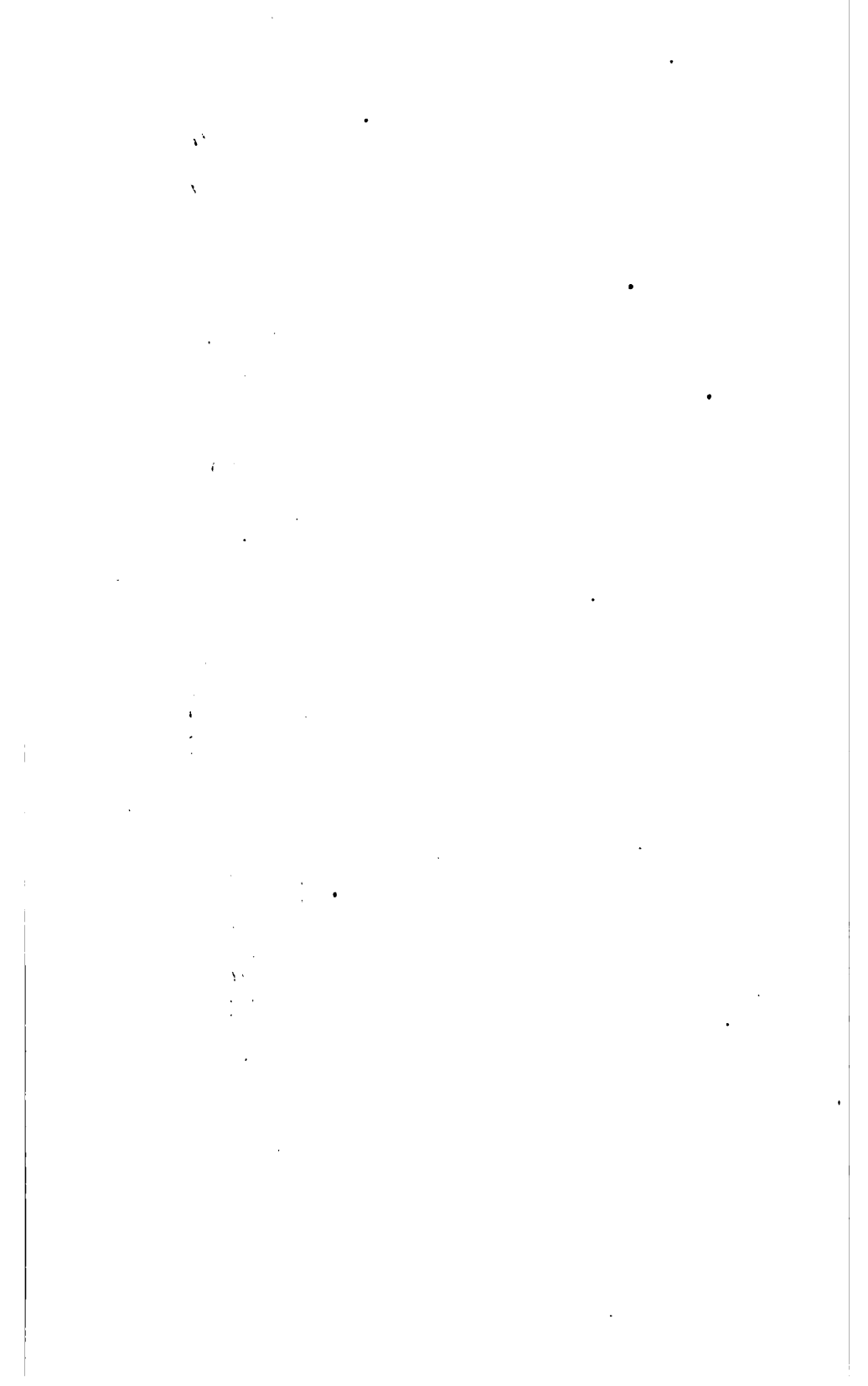
DATE 1912	NITROGEN	PHOSPHATES	CHLORINE	POTASSIUM	SODIUM
<i>October</i>	<i>grams</i>	<i>mgm. P.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>
26	10.73	874	800	823	166
27	8.39	717	533	629	105
28	12.48	1035	924	1282	466
29	8.89	744	382	694	140
30	12.68	1166	1560	1275	519
31	8.38	685	637	619	383
<i>November</i>					
1	10.36	560	1668	869	1143
2	5.08	171	312	166	80
3	14.63	832	1184	791	125
3 p.m.	7.17	475	629	685	75

The dog weighed 26.0 kilos. Parathyroidectomy was performed in the afternoon of October 31. Slight tremor was observed at 6 p.m., November 3. At 8.30 p.m., there was severe twitching and, at 9 p.m., the dog was killed. On the day of the operation there was a pronounced fall in the elimination of phosphorus. The usual amount of potassium was excreted and the output of sodium was greater than on any other day.

TABLE VI.
Urinary data. Dog 217.

DATE 1912	NITROGEN	PHOSPHATES	POTASSIUM	SODIUM
<i>November</i>	<i>grams</i>	<i>mgm. P.</i>	<i>mgm.</i>	<i>mgm.</i>
21	5.479	393	549	291
22	6.340	494	599	268
23	6.803	520	620	277
24	9.202	731	1133	702
25	6.571	533	757	218
26	7.352	242	320	53
27	6.750	310	235	21
27 p.m.	2.623	113	212	18

The dog weighed 11.0 kilos. The parathyroids were removed on the morning of November 25. At 3 p.m. November 27, the dog was in tetany and was exsanguinated. In this experiment the excretion of potassium and sodium paralleled that of phosphorus.



THE PHOSPHORUS CONTENT OF THE BLOOD OF NORMAL AND PARATHYROIDECTOMIZED DOGS.

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(Received for publication, March 19, 1913.)

As a result of previous experiments upon dogs,¹ in the course of which a marked retention of phosphorus was observed after removal of the parathyroid glands, it became of interest to ascertain the form in which phosphorus was stored in the organism under the experimental conditions. It is generally conceded that the tetany consequent upon parathyroidectomy strongly suggests an intoxication by a circulating toxin. In these experiments, therefore, the amount and distribution of phosphorus in blood and serum was investigated.

The experiments were conducted in pairs. After a few days' observation, during which period the dog was kept on a constant, weighed diet,² the parathyroids were removed. As soon as the twitching became well marked, the animal was exsanguinated. In the earlier experiments this was accomplished under ether anesthesia and from a carotid artery. Later, the dogs were bled from a femoral artery, using cocaine. The control dog, of the same sex (except in some of the earlier experiments), and of nearly the same size and age, was fed for a number of days on a diet that was, per kilo, the same as that of the experimental animal and was bled at the same interval after the last meal as was the parathyroidectomized dog. In this way it was hoped to eliminate the effect of age, sex, the absorption of food and of long-continued tetany.³

¹ Greenwald: *Amer. Journ. of Physiol.*, xxviii, p. 103, 1911.

² This never contained bone-ash. Infusorial earth was used in its stead.

³ For further details as to the conduct of the experiments see Greenwald: *loc. cit.*

For the estimation of phosphorus a slight modification of the Neumann⁴ method was used. It was found that, if Neumann's directions were followed, precipitation of the phosphomolybdate was not always complete. If, however, after oxidation was complete, the acid was diluted and neutralized with ammonium hydroxide, then acidified with nitric acid, heated to about 65°, complete precipitation occurred on addition of ordinary acid molybdate solution and digestion at 65° for one or two hours. The mixture was then allowed to stand at room temperature for several hours, ordinarily over night, then filtered on a Gooch crucible fitted with a piece of hardened filter paper and washed with cold water. The crucible and the precipitate were placed in the beaker in which the precipitation had been made and the precipitate was dissolved in a slight excess of standard sodium hydroxide solution (approximately seventh-normal). From 10 to 30 cc. of formalin,⁵ previously neutralized to phenolphthalein with sodium hydroxide, were added, then 10 or 25 cc. of standard acid and finally standard sodium hydroxide solution until the liquid was faintly alkaline to phenolphthalein. The volume of the alkaline solution required, less the volume of the acid used, multiplied by the factor, gave the amount of phosphorus in the sample. Even with less than 1 mgm. of phosphorus the error is only ± 2 per cent.

For the estimation of the different forms of phosphorus in blood and serum, several methods were tried. Most of these were found to be unsuitable. At first the blood was received in tared flasks containing glass beads and thoroughly shaken. The flasks were weighed and the contents transferred to a bottle containing several volumes of 95 per cent alcohol, recently distilled over sodium hydroxide. After standing a few days, the mixture was filtered through a linen bag and the residue extracted with hot alcohol in a continuous extraction apparatus. It was found that the extracts were always deeply colored and that they contained considerable quantities of material (hematin?) which made subsequent separation of the lipoids, either by precipitation with chloroform⁶ or by extraction with anhydrous ether, difficult and inaccurate.

⁴ Neumann: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 115, 1903.

⁵ Bang: *Biochem. Zeitschr.*, xxxii, p. 443, 1911.

⁶ Koch and Woods: *this Journal*, i, p. 206, 1906.

An attempt was made to dry the blood by mixing it with infusorial earth to form a thick paste and exposing this to the air, or *in vacuo*, at a moderate temperature (30–35°). A fine, dry powder was readily obtained but it was found that extraction with the usual solvent, removed only a small portion of the phospholipins known to be present.

For precipitation of the proteins and extraction of the lipoids, acetone was found to be most suitable. Addition of four volumes of acetone to blood or serum precipitates protein and inorganic phosphates completely. Subsequent treatment of the precipitate with hot acetone removes the lipoids practically completely but does not affect hemoglobin nor dissolve inorganic phosphate. If the acetone extract be evaporated and the lipoids precipitated with chloroform, only traces of phosphorus are found in the filtrate. After extraction with acetone, treatment with hot alcohol and ether removes, at most, only traces of phosphorus compounds from serum and only comparatively small amounts from whole blood.

For the determination of water-soluble phosphorus, direct extraction of the residue from the lipid extraction with hot water and dialysis were tried. Both were failures, removal of inorganic phosphate being incomplete within any reasonable period. Extraction with dilute hydrochloric acid was also unsuccessful, for the protein swelled to a jelly. Direct estimation of the non-colloidal phosphorus of serum was attempted in the filtrate from the kaolin precipitate produced by the method of Michaelis and Rona,⁷ but it was found that all of the inorganic phosphate added could not be recovered. Apparently some had been adsorbed.

The procedures that give the best results in the estimation of water-soluble phosphorus are the following:

1. The serum or blood is mixed with nine or nineteen volumes, respectively, of a solution containing 1 per cent of acetic acid and 0.5 per cent of picric acid. After a few hours the liquid is filtered and the phosphorus in an aliquot portion of the filtrate is estimated in the usual manner.

2. The dry residue from the lipid extraction is ground to a powder and treated with a dilute solution of hydrochloric acid (1

⁷ Michaelis and Rona: *Biochem. Zeitschr.*, vii, p. 329; viii, p. 356, 1908.

per cent by volume of the concentrated acid) saturated with picric acid. Ten or twenty volumes, respectively, of this solution are used for each volume of serum or blood under examination. The mixture is shaken at intervals and allowed to stand over night. It is then filtered and phosphorus is estimated in an aliquot portion of the filtrate.

Concordant values for the phosphorus precipitated from these extracts by barium and ammonium hydroxides were not obtained; consequently only total phosphorus was estimated.

That these methods are fairly accurate is shown by the figures given in tables I-III.

As already stated, separation of the phosphorus compounds of the blood into various fractions was not successful in the first series of experiments. The figures given in table IV were obtained by adding together the values obtained for the phosphorus in the different fractions. In the case of dog 111, however, the total phosphorus was estimated directly. As can readily be seen, the phosphorus content of the blood of the parathyroidectomized dogs was greater than in that of the control animals.

In the two following experiments, the blood was mixed with infusorial earth and dried. On subsequent extraction, phospholipins were not removed by the usual solvents. However, determinations of the total solids, nitrogen and phosphorus were made with the results indicated in table V. Six months later, samples of this dry powder were extracted with the picric-hydrochloric acid mixture and phosphorus estimated in the extract. The values obtained are given in the table.

The material for the next series of analyses consisted of defibrinated blood from a normal and a parathyroidectomized dog, obtained in the course of other experiments. Data relating to the sex, weight and food of the dogs are lacking. In this series the acetone extraction method was used but the residues were wasted in a vain attempt to remove inorganic phosphate by dialysis or by means of boiling water. However, direct estimations of the acid-extractable phosphorus were made, using the acetic acid-picric acid mixture already described. The figures obtained are given in table VI.

The blood from the next pair of dogs was allowed to clot. The serum was centrifuged to remove all the cells and then analyzed. Unfortunately, scarcity of material and a series of accidents made

it impossible to determine more than the total nitrogen and phosphorus and the phosphorus extracted by acetone and by subsequent treatment with absolute alcohol and ether. The figures obtained are presented in table VII.

Because parathyroidectomy was not followed by symptoms of parathyroid insufficiency, the next dog, 214, had both thyroids removed. Tetany then developed and the dog was bled. The blood, as also that of the control, was oxalated. In calculating the results of the analyses, which are summarized in table VIII, proper allowance has been made for the weight of the ammonium oxalate solution used.

In the three following experiments, perfectly clear serum, almost free from hemoglobin, was obtained. If the serum is at all red, it contains enough inorganic phosphate derived from erythrocytes to make analysis useless. The results obtained are summarized in table IX.

As can readily be seen on examining the tables, the total amount of phosphorus in the blood and serum of parathyroidectomized dogs is regularly greater than in normal dogs. This increase is not associated with a corresponding increase in the amount of total solids or of nitrogen; on the contrary it may be very marked in the presence of an equally pronounced diminution of total solids and nitrogen. The increase seems to be due largely, if not entirely, to an increase in that form of phosphorus which, for want of a better name, we have called acid-extracted phosphorus. A part of this fraction is inorganic phosphate; whether or not all of it is inorganic, cannot be stated. The amount of lipin-phosphorus in the blood and serum of dogs varies considerably and, apparently, bears no relation to the presence or absence of the parathyroid glands.

Very recently Juschtschenko⁸ has published the results of an investigation into the nitrogen and phosphorus content of the tissues of normal and parathyroidectomized dogs. Only his results with serum need concern us now. He dried the serum, first at 30°, then at 65°, and estimated inorganic phosphate by the method of Stutzer and Neumann. He states that the amount of inorganic phosphate of the serum is the same in dogs in tetany and in normal dogs. He makes no reference whatever to his

⁸ Juschtschenko: *Biochem. Zeitschr.*, xl, p. 64, 1912.

earlier paper⁹ in which he claimed that the blood of dogs and rabbits in tetany after thyroidectomy contained more inorganic phosphate than did the blood of normal animals. It is also interesting to note that, in the two instances in which he determined the phosphorus in the residue obtained after extraction of the lipoids from the dried serum, the higher values were obtained in the preparations from parathyroidectomized dogs. The conclusions drawn by Juschtschenko from his experiments are open to other objections. His dogs were in tetany for hours and days before they were bled so that it is impossible to distinguish the effect of the parathyroidectomy from the possible effect of the tetany and inanition. Phospholipins are apt to decompose when kept at 65° for twenty-four hours, as occurred in Juschtschenko's method of drying the serum. Apparently he did not attempt to test the accuracy of his method, to see if inorganic phosphate added to the serum could be recovered.

SUMMARY.

After removal of the parathyroid glands, the total phosphorus of the blood and serum of dogs is increased. This may be observed at a time when the tremor is still very slight. The increase may be as much as 160 mgm. of phosphorus per kilo of blood. The greater part of this increase is in the fraction which is insoluble in the usual lipid solvents but is soluble in a mixture of dilute hydrochloric or acetic and picric acids.

It is with great pleasure that I acknowledge my indebtedness to Dr. W. G. MacCallum, who was kind enough to remove the parathyroids from the animals required.

TABLE I.

Acid-extractable phosphorus in 25 cc. of ox-blood.

(Results expressed as cubic centimeters of standard solution required for titration of the phosphomolybdate.)

EXTRACTED WITH DILUTE ACETIC AND PICRIC ACIDS	EXTRACTED WITH HYDROCHLORIC AND PICRIC ACIDS, AFTER EXTRACTION WITH ACETONE
cc.	cc.
20.45	20.40
20.50	20.50
	20.50

⁹ Juschtschenko: *Zeitschr. f. physiol. Chem.*, lxxv, p. 141, 1911.

TABLE II.

Recovery, by the picric-acetic acid method, of inorganic phosphate added to blood.

($Mg_3P_2O_7$ dissolved in dilute HNO_3 , neutralized with NaOH before adding to the blood. Results expressed as cubic centimeters of standard solution required for titration of the phosphomolybdate.)

NUMBER	BLOOD	ADDED PHOSPHATE	BLOOD AND PHOSPHATE	PHOSPHATE RECOVERED
	cc.	cc.	cc.	cc.
1	15.17	49.85	64.00	48.83
	15.17			
2	20.45	49.85	70.50	50.02
	20.50	49.85	70.70	50.22
3		49.85	70.10	49.62
	26.4	66.0	92.2	65.8
	26.2	66.0	92.0	65.8

TABLE III.

Recovery, by the picric-hydrochloric acid method, of inorganic phosphate added to blood.

($Mg_3P_2O_7$ dissolved in dilute HNO_3 , neutralized with NaOH before adding to blood. Results expressed as cubic centimeters of standard solution required for titration of the phosphomolybdate.)

ACETONE-SOLUBLE PHOSPHORUS		ADDED PHOSPHATE	ACID-EXTRACTED PHOSPHORUS		PHOSPHATE RECOVERED
Blood	Blood and phosphate		Blood	Blood and phosphate	
cc.	cc.	cc.	cc.	cc.	cc.
11.45	11.60	49.95	20.40	69.60	49.20
	11.30	49.85	20.50	70.45	49.95
		49.85	20.50	71.33	50.83

TABLE IV.

Total phosphorus in blood from normal and parathyroidectomized dogs.

NUMBER	PARATHYROIDECTOMIZED	NUMBER	CONTROL
	mgm. per kilo		mgm. per kilo
105	477		
107	521	108	425*
110	512	109	416
111	474†		

* Parathyroids removed ten days before bleeding; no symptoms.

† Chronic latent tetany. See protocol of experiment below.

Dog 105. Fox-terrier, male, weight 10.70 kilos. The parathyroids were removed at 10 a.m., January 19, 1911. On the following day, slight twitching was noticed at 2 p.m. This was more marked at 3 p.m., when the dog was exsanguinated.

Dog 107. Mongrel, bitch, weight 9.20 kilos. Parathyroidectomy was performed at 2 p.m., January 31, 1911. Slight twitching was noticed at 2.30 p.m., February 4, and at 3.30 the dog was bled.

Dog 108. Mongrel, male, weight 8.35 kilo. Four parathyroids were removed on February 4, 1911. No symptoms appeared and the dog was bled at 3 p.m., February 14.

Dog 109. Bull-terrier, bitch, weight 8.30 kilos. Exsanguinated at 4.30 p.m., February 14, 1911.

Dog 110. Beagle, bitch, weight 9.60 kilos. Parathyroidectomy was performed at 3 p.m., February 10, 1911. Very slight twitching was noticed at 3.30 p.m., February 12. This gradually became more marked until, at 5.30 p.m., the dog was exsanguinated.

Dog 111. Bull-terrier, bitch, weight 13.0 kilos. Parathyroidectomy was performed on March 21, 1911, but no symptoms of parathyroid insufficiency appeared within the following two weeks. On April 6, a pup was born prematurely and died the same day. Another was born the following morning and lived about twenty-four hours. On the morning of April 8, a slight tremor was noticed. This grew more marked and continued several days. Two pups were found in the cage on the morning of April 10. They were quite well developed but the mother paid little attention to them and they died the following morning. On this day (April 11), four pups made their appearance. The mother suckled them but one died on the 16th and another on the 17th. The other two survived and were raised to an age of about four months when they were unfortunately lost. They seemed to be quite normal. The mother continued in tremor until April 21, two weeks in all. In June, attempts were made to induce tetany by feeding large quantities of meat and of extract of beef. These were not successful. After a return to the usual diet for three days, the dog was bled at 3 p.m.

TABLE V.

Analyses of blood from a parathyroidectomized dog (209) and from a normal dog (210).

	209	210
Total solids.....	17.80 per cent.	24.26 per cent.
Total nitrogen.....	2.31 per cent.	3.16 per cent.
Total phosphorus.....	592.7 mgm. per kilo.	430.3 mgm. per kilo.
Phosphorus extracted with di- lute acid.....	387.4 mgm. per kilo.	290.4 mgm. per kilo.

Dog #9. Bull-terrier, male, weight 13.50 kilos. The parathyroids were removed at 10.30 a.m., March 1, 1912. A slight tremor was noticed at 2.30 p.m., the following day. This became a little more marked during the afternoon but was not severe at 2.30 a.m., March 3. There was pronounced twitching at 10.20 a.m. and the dog was bled to death.

Dog #10. Bull-terrier, male, weight 13.00 kilos. Exsanguinated at 9 a.m., March 8.

TABLE VI.

Analyses of blood from a parathyroidectomized dog and a normal dog.

	PARATHYROIDECTOMIZED	NORMAL
Total nitrogen.....	3.02 per cent.	2.97 per cent.
Total phosphorus.....	482 mgm. per kilo.	409 mgm. per kilo.
Phosphorus extracted with acetone.....	145 mgm. per kilo.	135 mgm. per kilo.
Phosphorus extracted with alcohol and ether.....	11.7 mgm. per kilo.	12.2 mgm. per kilo.
Phosphorus extracted with picric-acetic acid solution...	284 mgm. per kilo.	239 mgm. per kilo.

TABLE VII.

Analyses of serum.

	PARATHYROIDECTOMIZED DOG 212	NORMAL DOG 213
Total nitrogen.....	9.833 gm. per kilo.	9.350 gm. per kilo.
Total phosphorus.....	209 mgm. per kilo.	179 mgm. per kilo.
Phosphorus extracted with acetone.....	129 mgm. per kilo.	133 mgm. per kilo.
Phosphorus extracted with alcohol and ether.....	Trace	Trace

Dog #12. Bull-terrier, bitch, weight 13.50 kilos. The parathyroids were removed at 11.30 a.m., June 17, 1912. At 4 p.m., the following day, the dog was in tetany and was exsanguinated.

Dog #13. Mongrel, bitch, weight 11.60 kilos. Bled at 4 p.m., June 22, 1912.

TABLE VIII.

Phosphorus in blood from a thyro-parathyroidectomized dog (214) and a normal dog (214B).

	MILLIGRAMS PHOSPHORUS PER KILO OF BLOOD	
	214	214B
Total.....	436	370
Extracted with acetone.....	162	140
Extracted with alcohol and ether.....	8	7
Extracted with picric-hydrochloric acid.....	233	192
Not accounted for (protein phosphorus?).....	33	31

Dog 214. Mongrel, male, weight 12.40 kilos. Parathyroidectomy was performed July 16, 1912. Tremor did not appear, nor was the excretion of phosphorus diminished. At 4.30 p.m., July 20, both thyroids were removed. At 11.30 p.m., the following day, there was slight, occasional twitching. This was more evident at 9 a.m. the next morning and the dog was bled to death.

Dog 214B. Mongrel, male, weight 13.50 kilos. Bled at 9 a.m., August 9, 1912.

TABLE IX.

Phosphorus in serum from parathyroidectomized and normal dogs.

NUMBER	PHOSPHORUS PER KILO OF SERUM			
	Total	Acetone extract	Acid extract	Residue
	mgm.	mgm.	mgm.	mgm.
Parathyroidectomized				
215	212	149	62.3	
217	222	128	76.7	
219*	291	194	87.7	3.8
Normal				
216	157	131	26.9	
218	244	186	44.4	2.4
220	167	110	45.5	

*Complete thyroidectomy.

Dog 215. St. Bernard, male, weight 26.0 kilos. The parathyroids were removed in the afternoon of October 31, 1912. Slight tremor appeared at 6 p.m., November 3. At 8.30, twitching was quite pronounced and the dog was bled.

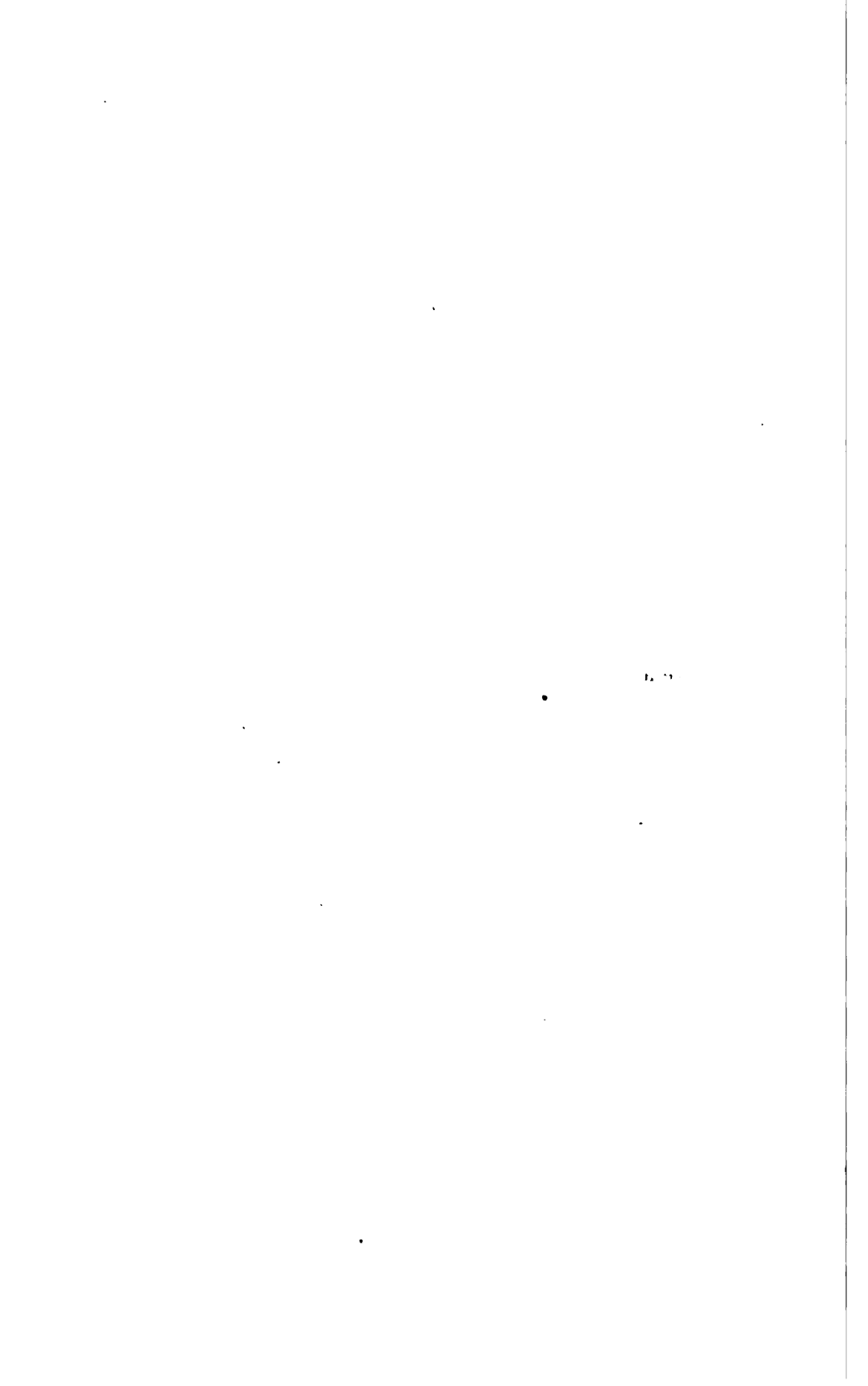
Dog #16. Mongrel, male, weight 14.0 kilos. Bled at 8 p.m., November 16, 1912.

Dog #17. Bull-terrier, male, weight 11.0 kilos. Parathyroidectomy was performed on the morning of November 25, 1912. Tremor was doubtful at 9 a.m., November 27 but, at 3 p.m., the dog had a severe attack of tetany and at 3.45 was exsanguinated.

Dog #18. Mongrel, male, weight 9.45 kilos. Bled at 4.30 p.m., February 12, 1913.

Dog #19. Mongrel, male, weight 14.7 kilos. Four parathyroids were removed on December 19, 1912. As no symptoms appeared, the right thyroid was removed on December 22. This was also without apparent effect and at 3 p.m., December 25, the other thyroid was removed. At 8 p.m., December 29, there was slight, though unmistakable, twitching. No change was observed at 10.30 p.m. and the dog was bled. A severe attack of tetany occurred during the bleeding.

Dog #20. Mongrel, male, weight 16.0 kilos. Bled at 10.30 p.m., January 12, 1913.



RESEARCHES ON PURINES.

ON 2-METHYLMERCAPTO-6,8-DIOXYPURINE AND 2-METHYLMERCAPTO-6-OXY-8-AMINOPURINE.

(TENTH PAPER.)¹

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(From the Sheffield Laboratory of Yale University.)

(Received for publication, March 21, 1913.)

The only method hitherto employed for the preparation of alkylmercapto purines has been to alkylate thiopurines by means of alkyl halides. Thus, Fischer² obtained 6-methylmercapto-7-methylpurine (I) by the action of methyl iodide on the potassium salt of 6-thio-7-methylpurine.

As far as we are aware no alkylmercapto-orthodiaminopyrimidines have hitherto been prepared.³ We have succeeded in preparing 2-methylmercapto-4,5-diamino-6-oxypyrimidine (V) and have used it for the synthesis of purine derivatives. The reactions leading to the preparation of this diaminopyrimidine are as follows: 2-Thio-4-amino-6-oxypyrimidine (II)⁴ was alkylated in a solution of potassium hydroxide at room temperature by means of dimethyl sulphate. This reaction had previously been carried out with methyl iodide but the reaction was not very smooth.⁵ The yield of 2-methylmercapto-4-amino-6-oxypyrimidine (III) obtained by the use of dimethyl sulphate was as high as 90 per cent of the calculated. This mercapto derivative then gave a quantitative yield of 2-methylmercapto-4-amino-5-nitroso-6-oxypyrimidine (VI) when acted on by nitrous acid. The nitroso compound was reduced smoothly to 2-methylmercapto-4,5-diamino-6-oxypyrimidine (V),

¹ Johns: this *Journal*, xiv, p. 1, 1913.

² Emil Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 437, 1898.

³ Some preliminary experiments on this work were made in 1906: Johnson, Johns and Heyl: *Amer. Chem. Journ.*, xxxvi, p. 172, 1906.

⁴ W. Traube: *Ann. d. Chem. (Liebig)*, cccxxxi, p. 71, 1904.

⁵ Johnson and Johns: *Amer. Chem. Journ.*, xxxiv, p. 181, 1905.

using ammonium sulphide as the reducing agent. The yield was 90 per cent of theory.

When 2-methylmercapto-4,5-diamino-6-oxypyrimidine was heated with urea it gave 2-methylmercapto-6,8-dioxypurine (IV), the yield being 60-70 per cent of the calculated. The mercapto group in this purine is very firmly bound but on boiling with a large excess of 20 per cent hydrochloric acid for twenty hours, methylmercaptan was evolved slowly and uric acid (VII) was formed.

It is well known that when orthodiaminopyrimidines are heated with thiourea 8-thiopurines are formed. Thus, when 2-thio-4,5-diamino-6-oxypyrimidine (IX)⁶ was heated with thiourea 2,8-dithio-6-oxypurine (XII)⁷ resulted. The presence of the methylmercapto group in position 2 instead of the sulphur renders 2-methylmercapto-4,5-diamino-6-oxypyrimidine less active towards thiourea. Instead of obtaining the expected 2-methylmercapto-6-oxy-8-thiopurine we obtained 2-methylmercapto-6-oxy-8-aminopurine (VIII). It is possible that traces of the 8-thiopurine were also formed although we did not detect any of this compound. The yield of the 8-aminopurine was 60 per cent of the calculated. This result may be explained by assuming that the thiourea formed guanidine thiocyanate which then reacted with the diaminopyrimidine. This view was confirmed by heating the diaminopyrimidine with guanidine thiocyanate which also gave 2-methylmercapto-6-oxy-8-aminopurine. The same result was also obtained by using ammonium thiocyanate instead of guanidine thiocyanate.

These results are interesting in view of the fact that the writer has attempted to obtain 8-aminopurines by heating other diaminopyrimidines with salts of guanidine but without success. Even when 2-thio-4,5-diamino-6-oxypyrimidine is heated with guanidine thiocyanate it fails to give an 8-aminopurine.

When 2-methylmercapto-6-oxy-8-aminopurine was heated with hydrochloric acid the amino group was removed before the methylmercapto group. On prolonged heating uric acid was obtained.

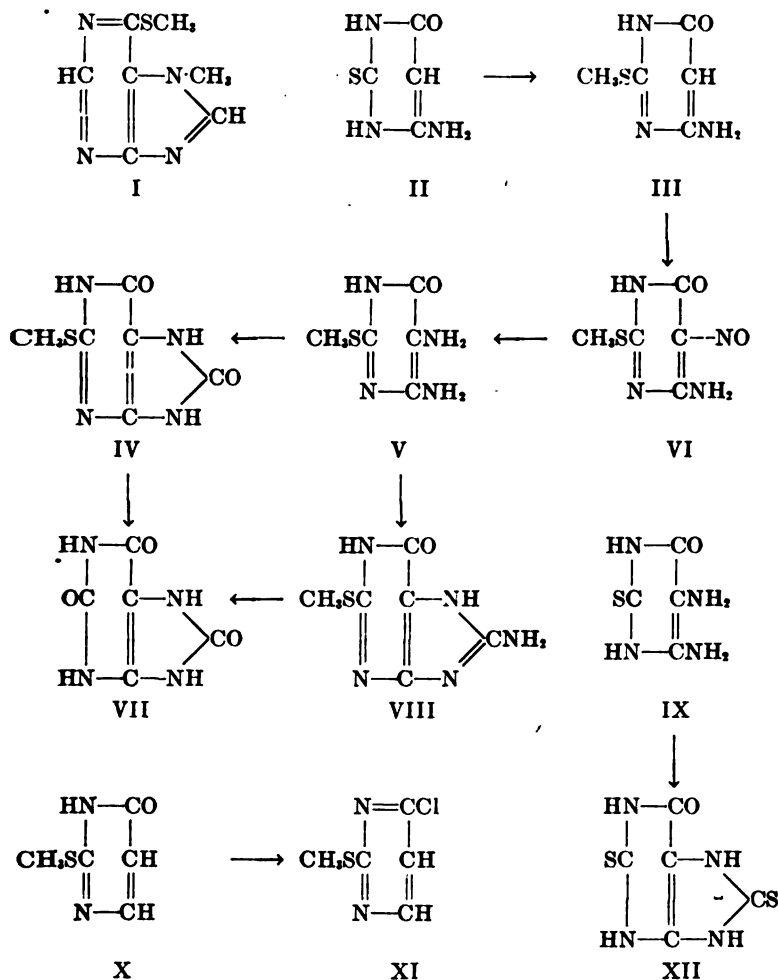
2-Alkylmercapto-6-oxypyrimidines (X)⁸ react readily with phosphorus halides to form 6-chlor derivatives (XI). We intend to

⁶ W. Traube: *Ann. d. Chem.* (Liebig), cccxxxi, p. 75, 1904.

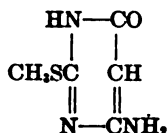
⁷ Johns and Hogan: *this Journal*, xiv, p. 299, 1913.

⁸ Wheeler and Johnson: *Amer. Chem. Journ.*, xxix, p. 496, 1903.

investigate the action of phosphorus halides on 2-alkylmercapto-6-oxypurines. Other researches on alkylmercapto purines are also in progress.

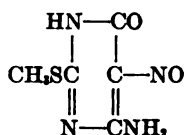


EXPERIMENTAL PART.

*2-Methylmercapto-4-amino-6-oxypyrimidine.*⁹

This compound was previously prepared by the action of methyl iodide on the sodium salt of 2-thio-4-amino-6-oxypyrimidine. We find that dimethyl sulphate can be used instead of methyl iodide. The yield is greater, the reaction requires less time and the cost is less. The methylation was performed as follows:

Twenty-five grams of pulverized 2-thio-4-amino-6-oxypyrimidine were dissolved in 100 cc. of a 10 per cent solution of potassium hydroxide, care being taken to avoid an excess of the alkali. Twenty-five grams of technical dimethyl sulphate were then added in small portions, with thorough shaking after each addition. In some cases it was found necessary to dilute with water as the precipitate which resulted became too thick to permit thorough mixing to take place. After the mixture had stood at room temperature for fifteen minutes it gave an acid reaction and the precipitate was filtered by suction. The mercapto pyrimidine thus obtained was removed to a flask while still moist, 200 cc. of 95 per cent alcohol were added and the mixture was heated to the boiling point of the alcohol. This dissolved most of the precipitate. The flask was then cooled and allowed to stand at room temperature for an hour. On filtering, 20 to 25 grams of pure 2-methylmercapto-4-amino-6-oxypyrimidine were obtained. This is 75 to 90 per cent of the calculated weight. When this was mixed with a pure sample of 2-methylmercapto-4-amino-6-oxypyrimidine obtained by alkylating with methyl iodide the melting point of the mixture was 267°C. This is the melting point of the pure substance.

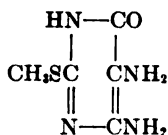
2-Methylmercapto-4-amino-5-nitroso-6-oxypyrimidine.

⁹ Johnson and Johns: *Amer. Chem. Journ.*, xxxiv, p. 181, 1905.

Twenty grams of 2-methylmercapto-4-amino-6-oxypyrimidine were dissolved in 350 cc. of water containing 5.1 grams of sodium hydroxide. A solution of 10 grams of sodium nitrite in 50 cc. of water was added. The mixture was then acidified by the gradual addition of 17 grams of glacial acetic acid. The precipitate which formed was white but turned blue in a short time. The mixture was allowed to remain at room temperature over night after which the precipitate was filtered off, washed with cold water and used, without drying, for the preparation of 2-methylmercapto-4,5-diamino-6-oxypyrimidine. The yield of the nitroso derivative was almost quantitative. It was but slightly soluble in hot water or alcohol and was not soluble in benzene. It formed a red solution in alkalies and blue in acids. A portion was purified for analysis by dissolving it in ammonia and precipitating with acetic acid. The substance did not melt but began to decompose at about 255°C.

	Calculated for $C_5H_7O_2N_4S$	Found:	
		I	II
N.....	30.10	29.75	30.16

2-Methylmercapto-4,5-diamino-6-oxypyrimidine.

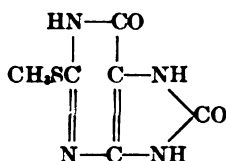


Fifty cubic centimeters of a 10 per cent solution of ammonium sulphide were placed in a 1-liter flask and heated on the steam bath. The moist 2-methylmercapto-4-amino-5-nitroso-6-oxypyrimidine obtained in the previous experiment was added gradually. Ammonium sulphide was also added when the solution turned red as this indicated that the nitroso compound was present in excess. When the ammonium sulphide was present in excess the solution was yellow. When all of the nitroso compound has been reduced the addition of excess of ammonium sulphide should be avoided or the diamino compound obtained will be highly colored. Sulphur separated after the reduction had proceeded for a few minutes. When the reduction was complete the mixture was concentrated by boiling in a casserole over a free flame until the separation of sulphur ceased. The solution was then filtered

rapidly to remove the sulphur. The filtrate on cooling gave an almost colorless, crystalline precipitate. A second crop was obtained by concentrating the mother liquor on the steam bath. The diaminopyrimidine was dried at 30–40°C. Drying at high temperatures causes it to become black. When the reduction was carried out carefully the diamino derivative was almost pure. It began to shrink at about 198°C. and melted with decomposition at 211°C. It was easily soluble in hot and appreciably soluble in cold water and moderately soluble in hot alcohol but was not soluble in boiling benzene. The yield based on the 2-methylmercapto-4-amino-6-oxypyrimidine used was over 90 per cent of the calculated.

	Calculated for $C_4H_6ON_4S$:	Found:
N.....	32.53	32.13

2-Methylmercapto-6,8-dioxypurine.



Five grams of urea and an equal weight of 2-methylmercapto-4,5-diamino-6-oxypyrimidine were intimately mixed by pulverizing together in a mortar. This mixture was heated in an oil bath at 165°C. for fifteen minutes and then at 170–180°C. until a hard cake had formed, about one hour in all. The reaction product was crushed and dissolved in hot dilute sodium hydroxide and the solution was clarified with blood coal. The purine was precipitated from the hot solution by acidifying with dilute hydrochloric acid. The yield was 3.5 grams or 60 per cent of the calculated weight. The purine was obtained in a finely divided, granular form. One part by weight required a little more than 100 parts of boiling water to effect solution. It was almost insoluble in alcohol, benzene, or glacial acetic acid. It did not dissolve readily in ammonia but was easily soluble in dilute sodium hydroxide. It dissolved with effervescence in concentrated nitric acid and gave the murexide reaction. It did not decompose at 320°C.

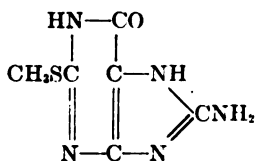
	Calculated for $C_6H_4O_2N_4S$:	Found:
N.....	28.28	28.17

Conversion of 2-methylmercapto-6,8-dioxypurine to uric acid.

One gram of 2-methylmercapto-6,8-dioxypurine was boiled with 150 cc. of 20 per cent hydrochloric acid under a return condenser for twenty hours. At first complete solution of the mercapto purine took place but after boiling for several hours uric acid began to precipitate. The reaction mixture was evaporated to dryness and the residue was dissolved in dilute sodium hydroxide, the solution filtered and acidified with hydrochloric acid whereupon uric acid precipitated.

	Calculated for $C_6H_4O_2N_4$:	I Found:	II
N.....	33.33	33.37	33.36

2-Methylmercapto-6-oxy-8-aminopurine.



This purine was obtained by heating one part by weight of 2-methylmercapto-4,5-diamino-6-oxypyrimidine with an equal weight of either thiourea or ammonium thiocyanate but was best prepared as follows:

Five grams of the mercaptodiaminopyrimidine and 5 grams of guanidine thiocyanate were pulverized together and the mixture was heated at 180°C. in an oil bath for two hours. During the heating the mixture melted partly, foaming ensued and, finally, a hard crust was formed. The reaction product was treated with cold water to remove an excess of guanidine thiocyanate, the residue was dissolved in dilute sodium hydroxide and the solution was clarified with blood coal. On acidifying the hot solution with acetic acid the purine was obtained as a fine powder. The yield was 60 per cent of the calculated. The substance did not decompose at 320°C. It dissolved in about 300 parts of boiling water. It was slightly soluble in hot alcohol or glacial acetic acid and insol-

uble in benzene. It did not dissolve very readily in ammonia. It gave a brilliant murexide reaction. When boiled with hydrochloric acid the amino group was hydrolyzed off before the mercapto group. Boiling with 10 per cent sodium hydroxide did not remove either the amino group or mercapto group.

	Calculated for $C_6H_7ON_4S$	Found:	
		I	II
N	35.53	35.44	35.67

When the above mercaptoaminopurine was boiled for twenty hours with 20 per cent hydrochloric acid, uric acid was obtained. Analyses on different samples resulted as follows:

	Calculated for $C_5H_4O_3N_4$	Found:	
		I	II
N	33.33	33.14	33.58

ON THE EXCRETION OF NITROGEN SUBSEQUENT TO LIGATION OF SUCCESSIVE BRANCHES OF THE RENAL ARTERIES.

By J. D. PILCHER.

(From the Pharmacological Laboratory, Western Reserve University Medical School.)

(Received for publication, March 22, 1913.)

MacNider¹ has shown that ligation of the posterior branch of the renal artery leads to necrosis of practically the posterior third of the kidney. This is followed by partial regeneration of the renal epithelium and glomeruli, reaching its height about the twenty-sixth to the thirtieth day; subsequently atrophy of the new tissue follows as a result of fibrosis of the stroma. Further, a collateral circulation develops in the area supplied by the ligated branch, first in the medulla, then at the cortex-medullary boundary zone and finally the cortex, usually in the form of vascular streaks. This collateral circulation takes place between the arteriolae rectae and pseudo-arteriolae rectae of the ligated and unligated vessels. Normally the pelvis and a portion of the renal papillae receive a partial blood supply through vessels entering the kidney with the ureter; there is a further anastomosis between the vessels of the capsule of the kidney and the recurrent suprarenal and phrenic arteries.

The following description is a report of two observations made as an attempt to determine whether there are any changes in the total nitrogen excretion incident to the renal changes. The effect of excision of various segments of the kidney has been studied by several observers, notably, Bradford,² Bainbridge and Beddard³ and Pearce.⁴ All agree that removal of three-fourths of the total

¹ W. de B. MacNider: *Journ. of Med. Res.*, xxiv, p. 425, 1911.

² J. R. Bradford: *Journ. of Physiol.*, xxiii, p. 415, 1898-9.

³ F. A. Bainbridge and A. P. Beddard: *Proc. Roy. Soc.*, lxxix, p. 75, 1907.

⁴ R. M. Pearce: *Journ. of Exp. Med.*, x, p. 632, 1908.

kidney substance results in death within a few weeks. In such animals Bradford found an increased output of nitrogen (as urea). This observation was not confirmed by other investigators, who were, therefore, unable to agree with Bradford's suggestion that the kidney has an influence on nitrogenous metabolism; but attributed the increased nitrogen excretion to inanition only. The present method, by minimizing complicating operative factors, offered a better chance of solving the effect of throwing a part of the kidney out of function.

Methods. The observations were made on one cat and one dog, both full-grown. The cat was fed hashed beef and the dog, ordinary dog biscuit, the nitrogen content of which was determined by the Kjeldahl method. The animals were kept in the usual metabolism cages. The urine was examined daily with the cat, and two or three times weekly with the dog, but daily, shortly before operations. The urines were preserved in strong sulphuric acid when kept longer than twenty-four hours. After determining the nitrogen excretion for suitable periods (two weeks with the cat, three to five with the dog) successive branches of the renal arteries were ligated under anesthesia⁵ as follows:

Ligations on the cat.

December 29, 1909. First operation: ligated branch of left renal artery.

January 12, 1910. Second operation, fourteen days later: ligated and cut the upper branch of the right renal artery.

January 26, 1910. Third operation, fourteen days later: ligated and cut the left renal artery, as the remaining branch could not be located. Following this operation the cat temporarily lost greatly in weight and ate but a fraction of its usual food, without evident cause (there seemed to be no sepsis). Observations were discontinued until the following winter.

February 9, 1911. Fourth operation, twelve months after ligating the left renal artery and thirteen months after the first operation, during which period the cat increased in weight from 3300 grams to 3800 grams: ligated and cut the lower branch of the right renal artery. The cat died in twenty-four hours, after severe nausea, vomiting, great prostration and subnormal temperature. At autopsy no peritonitis was present; the left kidney was atrophied (the artery was ligated one year previously); the right kidney

⁵ The dog was etherized; the cat received 3 cc. per kilogram of the following solution *per rectum* (supplemented by ether): atropine sulphate, 0.02 gram; morphine sulphate, 1.0 gram; urethane, 20.0 grams; aqua, 100 cc.

was adherent to the abdominal wall, but the capsule was very easily stripped from the cortex; it was practically normal in size, with the upper pole somewhat smaller than the lower (the upper renal branch had been ligated thirteen months previously).

Ligations on the dog.

April 17, 1910. First operation: ligated lower branch of the left renal artery. Following the operation there was a local infection and considerable prostration. Recovery was complete in five or six weeks.

June 24, 1910. Second operation, seventy days later: ligated one branch of right renal artery; this operation was also followed by considerable prostration, without infection, and observations were discontinued.

December 5, 1910. Third operation, seven months after the first and five months after the second: ligated upper branch of left kidney. Following the operation there was severe prostration similar to the cat, as described above; death in forty-eight hours. At autopsy there was no peritonitis; the right kidney was atrophied but there was one small patent arterial branch running to it; the left kidney was normal in size but the lower pole was somewhat smaller than the upper.

Table of ligations and N intake and output (in urine).

LIGATION OF BRANCHES OF RENAL ARTERIES	TOTAL KIDNEY SUBSTANCE WITH NORMAL BLOOD SUPPLY	TOTAL N		
		Intake <i>grams</i>	Output <i>grams</i>	Retention <i>per cent</i>
<i>Dog.</i>				
	4/4	4.46	3.77	15.4
Left lower branch.....	3/4	4.46	3.75	15.0
Entire right artery.....	1/4	4.46	3.25	27.1
<i>Cat.</i>				
	4/4	3.15	3.25	-3.1
Left branch.....	3/4	3.18	3.22	-1.2
Right branch.....	1/2	2.96	2.86	3.3
Entire left artery.....	1/4	2.88	2.79	3.1

The nitrogen excretion in the cat. The average daily excretion was determined during the different periods of observation, each of fourteen days' duration. As is shown by the accompanying table the excretion remained practically unchanged after the first ligation, the daily output exceeding the intake by 0.04 gram. Subsequent to the second ligation, that is, with one branch of each

renal artery ligated, the second excretion was but 0.10 gram (3.5 per cent) less than the intake. One entire renal artery was then ligated, leaving the kidney tissue supplied by but one (of a total of four) arterial branch. As was stated above, this resulted in severe prostration and anorexia, the daily nitrogen output (2.33 grams) exceeding the intake (1.58 grams) by 0.75 gram, or about 30 per cent. The severe prostration and markedly increased nitrogen excretion corresponds quite closely to the observations of others, mentioned above, on removal of three-quarters of the total kidney substance. However, in this case the animal made a perfect recovery after a few weeks, and it may be that ligation of one entire renal artery and one branch on the opposite side did not destroy the function of quite three-quarters of the total renal substance; or there may have been a development of a collateral circulation from the capsule or between the smaller vessels of the ligated and unligated branches, which was sufficient to preserve complete loss of function of the area nourished by the ligated branch. With the establishment of the collateral circulation there may have been regeneration of renal tissue in case of atrophy immediately subsequent to the ligation. During a period of twelve days, one year later, the cat was practically in nitrogen equilibrium, the daily intake exceeding the output by but 0.09 gram. Inasmuch as death ensued so promptly (in about twenty-four hours) on ligation of the last remaining arterial branch it follows that, in this case, occlusion of one branch of the renal artery does not result in sufficient collateral circulation from the capsule to preserve the function of the ligated area, so that, after one year, it is unable to preserve life, even for a short time, if the renal artery is then ligated.

The nitrogen excretion in the dog. The nitrogen intake remained constant throughout the periods of observation (4.46 grams daily). There was a deep-seated infection following the first and considerable *prostration* after the second ligation⁶ so that observations were not made until the dog returned to its normal condition. One month after the first ligation the dog was in normal condition and during a period of thirty-five days, excreted the same daily quantity of nitrogen (3.75 grams) as before operation. Dur-

⁶ At this period probably one entire artery and one branch of the opposite artery had been ligated.

ing this period there was a 3.5 per cent gain in weight as against a pre-operative gain of 1.7 per cent. The next observations were made during a period of thirty-seven days, four months after the second operation, at a time when one kidney had practically atrophied and one branch of the opposite side had been ligated for about seven months; the average daily nitrogen excretion fell 13 per cent below the normal excretion. During this period (37 days) the dog lost about 3.5 per cent in weight. With considerably less than one-half of the normal kidney substance functioning the dog was apparently in perfect condition and able to excrete approximately (13 per cent less) the normal quantity of nitrogen; perhaps there was some deficiency in nitrogen excretion since there was a retention with a loss in weight.

Inasmuch as the dog died within forty-eight hours after ligation of the last renal arterial branch, and without sepsis, it can be deduced that, during the seven months' interval between the ligation of the two branches, as in the cat, there was not formation of sufficient collateral circulation from the capsule to preserve the function of the ligated areas.

The profound prostration following the second ligation was undoubtedly due to removing the function of the entire kidney for this kidney was atrophied at autopsy, so that practically but one-fourth of the entire kidney substance was functioning at this time (as one branch of the opposite side had previously been ligated). This prostration was probably accompanied by a marked temporary nitrogen excretion, as was noted in the cat under similar conditions and observed by others when three-fourths of the kidney substance was removed at one time.

The daily nitrogen excretion in the cat immediately following the ligation. Subsequent to the first two ligations the output was either normal (after the first) or somewhat below normal for a day or two; then for a period of four or five days the output was increased to 13 per cent (first operation) and 11 per cent (second operation) above the intake; in each case during the eight days until the next operation the output fell slightly below the intake. Following the third ligation (one entire artery) the excretion was increasingly greater than the intake during the observation period of fourteen days, when the animal lost weight and the nitrogen intake was below normal.

Studying the influence of ether anesthesia upon the excretion of nitrogen in dogs, Hawk⁷ reports an increased nitrogen output following the anesthesia which may persist for several days. As the results in the cat agree with Hawk's observations on dogs, the temporary increased nitrogen excretion following the ligation may be attributed to the anesthetic in part at least.

The quantity of urine. With the greater part of the kidney substance removed, Bradford's dogs excreted permanently a volume of urine much above the normal, and of low specific gravity. Bainbridge and Beddard were unable to confirm his results but report the usual quantity and specific gravity of urine. The results in this series agree with those of the latter observers. The dog excreted practically the same volume of urine throughout, from 100–150 cc. daily; no observations on the total volume of the cat's urine were made until after the second operation when the quantity remained constant (75 cc.) until the final operation (75 cc. is not an abnormally large quantity of urine for a cat of 3300 grams). At times immediately following operation the water intake and output were increased in quantity.

Just before the final operation the urine of both animals was free from albumen and casts of any kind.

Partial nephrectomy in dogs has been reported to be followed by considerable rise in blood pressure;⁸ it would seem that such increased pressure probably would be associated with cardiac hypertrophy. In this work no blood pressure observations were attempted; however, it might be inferred that such marked loss of kidney substance would result in increased blood pressure and cardiac hypertrophy. Such hypertrophy did not occur in the cat. Joseph⁹ gives the ratio of the heart weight with the body weight in 26 cats as 0.45 per cent; in this laboratory a similar average in 20 cats gave 0.40 per cent. The ratio in the cat under discussion was 0.44 per cent. The dog reported had a fairly large goitre so that similar observations have little value. (However, the ratio was markedly increased—1.44 per cent as compared with 0.743 per cent reported by Joseph.)

⁷ P. B. Hawk: *this Journal*, iv, p. 321, 1908.

⁸ T. C. Janeway: *Soc. of Exp. Biol. and Med.*, vi, p. 109, 1908.

⁹ D. R. Joseph: *Journ. of Exp. Med.*, x, p. 521, 1908.

CONCLUSIONS.

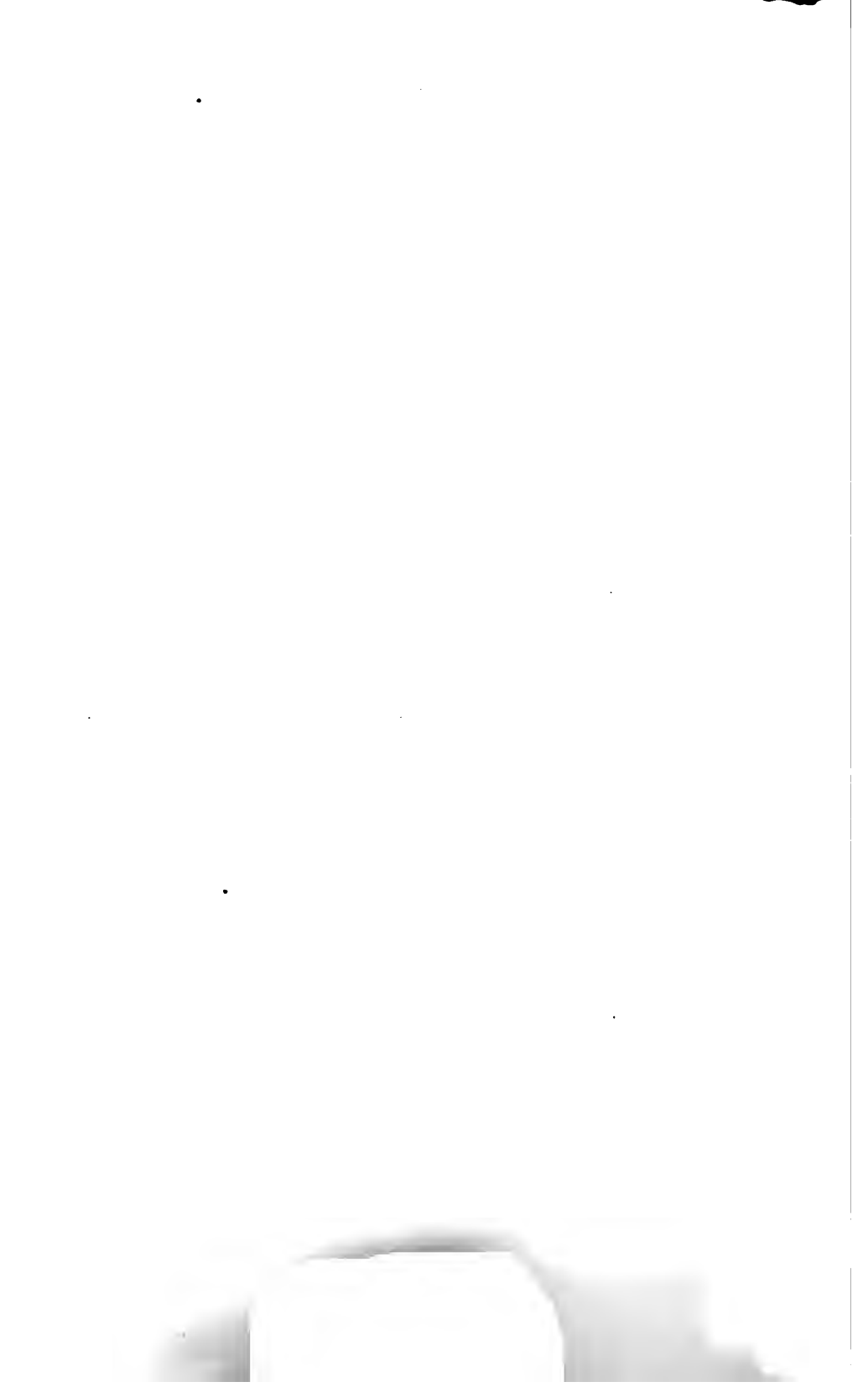
Ligation of one of the branches of both renal arteries, *i.e.*, approximately half of the blood supply, does not cause any noticeable disturbance in renal function. The urine and the nitrogen excretion remain practically normal, with a slight tendency to nitrogen retention, which is probably not accidental.

Successive complete ligation of one renal artery and one branch of the other (*i.e.*, shutting off three-fourths of the arterial supply) results in marked temporary prostration, anorexia, and loss of weight, with nitrogen output much greater than the intake. The animals recover gradually to a condition similar to that when but one-half of the arterial supply was ligated. On the assumption (justified by MacNider's histological data) that the ligated areas took no part in urine formation, the remaining one-fourth of the kidney was able to secrete the urine almost as effectively as the entire kidney area.

Occlusion of one branch of the renal artery does not result in sufficient collateral circulation from the capsule to preserve the function of the ligated area; so that even after twelve months it is unable to preserve life if the renal artery is then ligated.

With but one-fourth of the kidney substance functioning the quantity of urine was practically normal; the urine contained no albumen or casts; cardiac hypertrophy did not occur.

I take pleasure in acknowledging my indebtedness to Professor Sollmann for his aid during this work.



ON THE IODINE AND PHOSPHORUS CONTENTS, SIZE AND PHYSIOLOGICAL ACTIVITY OF THE FETAL THYROID GLAND.

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(From the Research Laboratory in Organotherapeutics of Armour and
Company, Chicago, Illinois.)

(Received for publication, March 31, 1913.)

In two previous communications¹ it was shown that fetal thyroid tissue possesses a strong selective affinity for iodine, that the glands of beef fetuses contain an appreciable amount of iodine as early as the third month of intra-uterine life, or approximately six months before maturity, and that the iodine content increases proportionately with the age of the fetus. It has also been conclusively demonstrated that the presence of active principle in a gland is an indication of its relative physiologic activity and that consequently the fetal thyroid is functionally active long before maturity of the fetus. No further definite conclusions could be drawn at that time because of the lack of raw material. During the past year at various seasons the glands from 705 beef fetuses six to nine months old have been secured and analyzed within a short time after collection and the results of the determinations are given in the accompanying tabulation.

It is generally assumed that the thyroid of the human fetus is functionally active at the end of the sixth month of intra-uterine life,² although previous investigators have been unable to find iodine in the gland whether of human or animal origin at this early stage in sufficient quantities to support these clinical observations. This in turn has led to the erroneous statement found in text-books on physiological chemistry that the thyroid glands of new-born animals do not contain iodine. The material available from normally healthy human fetuses is, of course, very limited and since the time of gestation for cattle is nine and one-half

¹ This *Journal*, xi, p. 489, 1912; xii, p. 55, 1912.

² I. Ott: *Textbook of Physiology*, 1909, p. 403.

months, or approximately the same as in the human race, it was decided to use for this investigation fetal thyroids from these animals ranging in age from six months after conception until a few weeks before delivery.

If the gland is active and secreting during this last three months' period before birth, we should expect to find iodine present in amounts and proportions similar to those existing in the glands after birth simply because our present knowledge of the chemistry of the thyroid would make it very difficult, if not impossible, to accept any theory of a physiologically active gland not associated with iodine, whether in fetus, suckling, young or full-grown animal.

In collecting the materials the following procedure was used. The throat of the fetus was opened and the oesophagus with the adherent thyroid removed. The whole gland, including both lobes and the isthmus, was separated very carefully, freed from connective and other tissue, rinsed with water and dried with a soft, absorbent cloth. The glands were then weighed separately and the figures given in the tabulation indicate, therefore, the average total weight of the entire fetal gland.

The term goitrous gland, as applied in this paper, refers only to adult thyroids weighing 150 grams or over, while the disproportionately large fetal glands are referred to simply as enlarged. The analytical data obtained and the morphologic observations made in the course of this investigation may, however, throw some light on several phases of the goitre situation in cattle as it occurs in the United States.

It was found that the individual fetal thyroids varied enormously in size and materially in color. It did not seem advisable to use the difference in color as a means of separation, so the glands were divided into two groups according to size. Glands weighing less than 20 grams were considered as normal and treated separately, while those weighing 20 grams or over were classified as large. Wherever possible, the glands were further subdivided according to sex.

After weighing and sorting, the various lots were finely minced, desiccated at a temperature not exceeding 65°C. to constant weight in shallow porcelain dishes and freed from fat by means of petroleum ether in a Soxhlet extractor. The dry fat-free material, ranging in color from a light gray to a dark reddish brown, corresponding to the different shades of the fresh glands, was pow-

dered in a porcelain mortar to pass a forty-mesh sieve and the various determinations made in duplicate on this material.

For comparative purposes the iodine and phosphorus contents of desiccated thyroid glands from adult animals are given in the tabulation. Each sample represents several hundred animals and was collected during the same period as the corresponding lots of fetal glands. They give, therefore, a fair idea of the average thyroid activity of the species at the various seasons. It was noticed that fresh beef thyroids, containing 0.30 per cent or more of iodine on the dry fat-free basis, both from fetuses and adult animals, possessed a peculiar, pungent, garlic-like odor resembling both that of phosphine and of phosphorus while undergoing oxidation. The odor was especially pronounced when the glands were cut open, while they still retained the animal heat, and disappeared gradually upon chilling and completely during the desiccation process. It was never observed in goitrous or normal glands low in iodine. In no instance, however, could phosphorescence be detected in the raw gland, although carefully watched for. The usual preliminary and identification tests for free phosphorus, free iodine and hydrogen sulphide were all negative. It was, therefore, concluded that the substance causing the odor, if an iodine-phosphorus compound, must be an organic complex. That it is associated with or entirely dependent upon the quantity of iodine in thyroid combination is, however, very apparent. When the percentage of iodine is high the reaction evidently is carried on in the gland proper some time after the blood stream ceases to circulate the secretions and the accumulation becomes sufficient to produce the characteristic odor when the tissue is exposed. A similar garlic-like odor was noticed in a certain lot of fresh sheep thyroids which afterwards proved to contain 0.53 per cent of iodine on the dry basis, an exceptionally high amount for American sheep glands.

In attempting to reach somewhat more definite conclusions concerning the relationship between the phosphorus and iodine contents of the thyroid, total phosphoric acid determinations were made on the desiccated, fat-free glands according to the official volumetric method³ in addition to the iodine which was estimated by the Hunter method.⁴

³ Bulletin 107 (Revised), Bureau of Chemistry, 1908.

⁴ A. Hunter: this *Journal*, vii, p. 321, 1910.

Fetal Thyroid Glands

		TOTAL NUMBER OF FETAL GLANDS	NUMBER OF NORMAL FETAL GLANDS	AVERAGE WEIGHT OF NORMAL FETAL GLANDS	IODINE IN DEMON- STRATED FAT-FREE GLANDS	PHOSPHORUS IN DEMON- STRATED FAT-FREE GLANDS
				grams	per cent	per cent
March, 1912.	Mixed glands: both sexes ...	10	7	8.0	0.174	0.59
March, 1912.	Mixed glands: both sexes ...	33	24	9.0	0.190	0.73
August, 1912.	Mixed glands: both sexes ...	6	6	9.1	0.322	lost
August, 1912.	Mixed glands: both sexes ...	15	14	8.6	0.174	0.73
September, 1912.	Male glands...	15	15	9.0	0.275	0.62
	Female glands.	7	5	8.6	0.294	0.59
September, 1912	Male glands...	14	10	10.5	0.173	0.70
	Female glands.	14	9	7.0	0.282	0.66
December, 1912.	Male glands...	15	14	5.8	0.258	0.63
	Female glands.	16	15	6.4	0.327	0.64
December, 1912.	Male glands...	21	21	7.4	0.296	0.69
	Female glands.	28	28	7.1	0.319	0.67
January, 1913.	Male glands...	14	14	10.4	0.232	0.63
	Female glands.	24	20	8.6	0.401	0.66
January, 1913.	Male glands...	42	34	11.7	0.201	0.65
	Female glands.	47	42	9.3	0.307	0.69
January, 1913.	Male glands...	33	26	8.6	0.211	0.62
	Female glands.	29	21	9.6	0.211	0.73
January, 1913.	Male glands...	25	15	12.2	0.175	0.66
	Female glands.	27	18	11.1	0.181	0.62
January, 1913.	Male glands...	18	15	8.9	0.222	0.61
	Female glands.	21	19	10.3	0.248	0.59
February, 1913.	Male glands...	38	30	9.8	0.187	0.66
	Female glands.	41	25	9.0	0.226	0.65
February, 1913.	Male glands...	45	30	11.1	0.164	0.76
	Female glands.	43	27	12.3	0.168	0.69
March, 1913.	Male glands...	35	21	12.4	0.159	0.76
	Female glands.	29	16	11.0	0.223	0.69
Total.....		705	541	9.6		

NUMBER OF LARGE FETAL GLANDS	AVERAGE WEIGHT OF LARGE FETAL GLANDS	IODINE IN DESIC- CATED FAT-FREE GLANDS	PHOSPHORUS IN DESI- CATED FAT-FREE GLANDS	APPROXIMATE NUM- BER OF MIXED ADULT GLANDS	AVERAGE WEIGHT OF ADULT GLANDS	IODINE IN DESIC- CATED FAT-FREE GLANDS	PHOSPHORUS IN DESI- CATED FAT-FREE GLANDS
	grams	per cent	per cent		grams	per cent	per cent
3	30.0	0.025	0.78	400	59.0	0.053	0.85
9	23.0	0.050	0.82				
				450	22.0	0.327	0.49
1	22.8	0.019	0.78				
2	35.7	0.020	0.76	400	23.4	0.300	0.51
4	48.7	0.025	0.87				
5	81.0	0.014	0.89				
1	71.8	0.013	1.01				
1	32.1	lost	lost	800	32.1	0.239	0.66
4	40.4	0.024	1.05				
8	31.9	0.047	0.67				
5	24.7	0.120	0.71				
7	48.2	0.006	0.93	800	28.8	0.197	0.63
8	30.8	0.056	0.77				
10	41.7	0.011	0.81				
9	28.7	0.041	0.76				
3	47.3	0.011	0.75				
2	26.6	0.108	0.71				
8	45.8	0.019	0.94				
16	47.6	0.028	0.83	850	44.3	0.127	0.72
15	46.5	0.017	0.99				
16	45.2	0.025	1.00				
14	32.0	0.030	0.96	450	49.2	0.111	0.80
13	36.2	0.032	0.95				
164	40.0			4150	36.2		

The total average weight of the normal fetal glands is 9.6 grams with a maximum of 19.8 grams and a minimum of 1.7 grams. The large glands show an average of 40.0 grams, maximum of 194.0 grams and a minimum of 20.2 grams, while the average weight of the adult thyroids is only 36.2 grams.

Before discussing the tabulated results the following data and observations in regard to age, size, weight and sex of the various fetuses and adult animals should be given.

The fetuses, when collected for analysis, were taken at random from the supply at hand at different times. In some lots younger fetuses predominate while in others nearly mature fetuses are in the majority. It was, however, decided to analyze the lots as they came along without any further distinctions than those already described.

As stated above, the range of ages of the fetuses is from six to nine months and this naturally exerts a proportional influence on the size of the fetuses. The several breeds of cattle also differ materially. The weights of all the individual fetuses were not recorded when the thyroid glands were removed; this was due to lack of facilities, but it was noticed that fetuses possessing enlarged glands, although otherwise apparently well proportioned, invariably were smaller than the fetuses with normal glands. This feature was very striking. The fetuses used in this investigation weighed from 20 to 100 pounds.

The possible influence of castration upon the iodine content of the thyroid glands is, of course, eliminated as far as fetuses are concerned, whereas a large percentage of the adult animals used for comparison have been subjected to this operation.

By comparing the figures in the tabulation it will be seen that the amounts of iodine in the normal fetal glands, in spite of their fewer numbers and considerable unevenness in size and age, are much more uniform throughout the entire year than those of the thyroids from full-grown animals and it seems apparent that the seasonal change in iodine content, which is so evident in the healthy glands from mature animals,⁵ is not pronounced in the normal fetal thyroid.

Normal fetal glands are relatively larger and contain more iodine and phosphorus per unit of body weight than thyroids from fully

⁵ Seidell and Fenger: *this Journal*, xiii, p. 517, 1913.

mature animals. As would be expected the male glands are somewhat larger than the female thyroids, the average weight being 9.9 grams for the male and 9.3 grams for the female, but it should also be noted that the smaller female glands contain a higher percentage of iodine than the male glands. As proportions and sizes of these glands correspond fairly well with the differences in body weight of the two sexes, the suggestion presents itself that the female metabolism requires more thyroid activity according to body weight than the male during the fetal stage as well as in the animal after birth.

A most striking feature of the tabulated results is the high percentage of abnormally large fetal glands. This enlargement affecting both lobes and the isthmus is quite pronounced at the third or fourth month of intra-uterine life, increasing proportionately with the growth of the fetus. It is, therefore, very evident that a large percentage of these animals pass through the fetal stage of life with exceptionally large glands low in iodine content.

It is generally understood that a certain number of goitrous glands prevail throughout the year in fully mature cattle, but observations indicate that climatic conditions, at least in the United States, exert a decided influence on the growth and that the enlargement affects a considerably higher number of adult animals during the winter and spring, than in summer and fall. The goitrous glands from full-grown cattle, which have come under observation during the last eighteen months, ranged in weight from 150 to 400 grams with average iodine and phosphorus contents of 0.016 per cent and 0.85 per cent respectively. These glands were all obtained in the winter and spring months and although beef thyroids in numbers of from 25 to 100 were collected twice a week during all this period of eighteen months, no goitrous glands were encountered in the summer and fall seasons. The percentage of goitrous glands in grown animals even during the cold season does not begin to compare with the proportion of enlarged glands found in the fetuses.

It should be stated here that the possibility is not entirely eliminated that the pregnant animals whose fetuses show enlarged glands all come from goitrous regions of the country, but if that holds true we naturally should expect to find a similar high percentage of goitrous glands in the general run of adult animals, and this is certainly not the case.

It has not been definitely determined or verified whether the enlargement of the thyroid gland, which affects so many of the fetuses, disappears rapidly after birth or persists for some time in the young animals. At present, however, observations indicate that, next to the fetuses, large glands are most frequently met with in sucklings and that there is a gradual decrease in the percentage of affected glands as the animals mature.

The fact that so many fetal glands are disproportionately enlarged, while the adult glands remain undisturbed, makes it very difficult to explain the fetal enlargement simply as a goitrous infection resulting from the ingestion of toxic food or water by the pregnant animal. At the same time the general appearance of these large fetal glands and their low iodine and high phosphorus contents coincide so closely with the corresponding features of goitrous glands from mature animals that the resemblance is very striking. This discrepancy may be explained by concluding that the demand for iodine in the rapid fetal metabolism and growth, in certain instances, may exceed the available supply furnished by the pregnant animal. This supply may be sufficient for the maintenance of the maternal metabolism leaving the adult thyroid normal, but not sufficient to prevent iodine starvation and enlargement of the fetal gland.

Another factor of considerable importance, shown by the tabulated data, is the relative proportion of iodine to that of phosphorus in the various glands. In spite of the size the enlarged glands contain less total iodine and much more total phosphorus than the normal thyroids. This peculiar condition holds true in case of fetal as well as adult glands, and is not specific to beef thyroids only, as it has been identified, for instance, in all goitrous hog and sheep thyroids which have so far come under observation. Since accumulation of organic phosphates does not occur in normal glands of average iodine content, the idea readily suggests itself that iodine in thyroid combination may be a factor of vital importance in the phosphorus metabolism of the body.

It may be mentioned that the work of collecting this raw material is time-consuming and often connected with considerable difficulties, as the fetuses are not plentiful the year round and are almost unobtainable at certain seasons. It is furthermore impossible to obtain systematic information concerning the sources and

localities from which the various animals are derived, thus further **masking** the evidence and making it more difficult to collate the **real facts** into a conclusive whole.

SUMMARY.

It has been demonstrated conclusively that functional therapeutic activity and presence of iodine coincide in the fetal thyroid during intra-uterine life in analogy with the conditions existing during extra-uterine life.

The iodine content of the normal-sized fetal thyroid glands during the last three months of intra-uterine life seems to be fairly uniform throughout the various seasons.

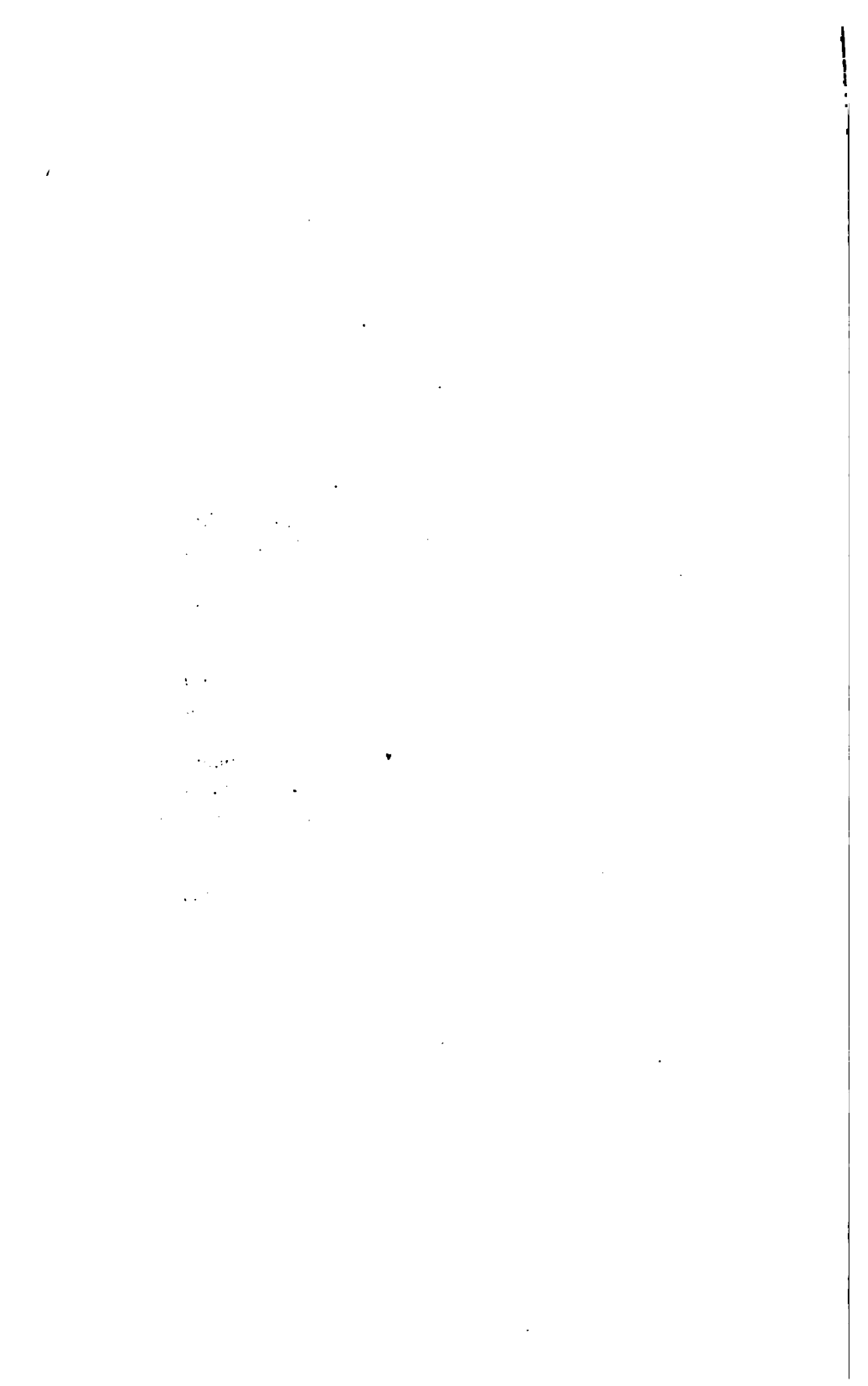
Normal fetal glands are relatively larger and contain more iodine and phosphorus per unit of body weight than thyroids from fully mature animals.

The normal female fetal thyroid glands show a higher content of iodine and seem, therefore, to possess greater functional activity than the male fetal glands.

Fetuses possessing enlarged thyroid glands on the whole were considerably smaller than average fetuses of the same age with normal thyroids.

The enlargement of fetal thyroids exceeds, both in frequency and size, the number of goitrous-affected glands of fully mature animals and is apparently the consequence of insufficient supply or faulty assimilation of iodine on the part of the pregnant animal.

Enlarged glands in general, both fetal and adult, contain less total iodine and much more total phosphorus than normal thyroids.



THE UTILIZATION OF AMMONIA IN THE PROTEIN METABOLISM.

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It has long been the opinion of the physiological chemist that the higher animal organism was dependent upon the amino-acids for the maintenance of protein anabolism. That plants of all orders, from the lowest to the highest, are able to synthesize protein from the salts of ammonium is, of course, fully established. For the animal organism, however, the prevailing opinion seemed conclusively established to the effect that the building-stones of protein could not be synthesized from ammonia and fatty acids. Formulated in chemical terms, the animal organism was not held to possess the power of effecting the replacement of one hydrogen attached to the α -carbon of a fatty acid by an NH_2 radical to form an α -amino-acid.

Ammonium introduced into the body from without, formed within the tissues through reactions of metabolism, or formed within the intestinal tract by the action of bacteria, was held to be converted into urea or eliminated as such. It was not held to be converted into amino-acids.

Recently published experiments by Knoop,¹ Embden² and their confrères have brought evidence to the effect that the animal body does possess the power of converting aliphatic and aromatic α -ketonic acids into amino-acids. This has led Grafe and Schläpfer³

¹ Knoop: *Zeitschr. f. physiol. Chem.*, lxvii, p. 489, 1910.

² Embden: *Biochem. Zeitschr.*, xxix, p. 423, 1910.

³ Grafe and Schläpfer: Über Stickstoffretention und Stickstoffgleichgewicht bei Fütterung von Ammoniaksalzen., *Zeitschr. f. physiol. Chem.*, lxxvii, p. 1, 1912.

and Abderhalden⁴ to study the effect of ammonia on the protein metabolism of dogs. Grafe and Schläpfer came to the conclusion that dogs on a high carbohydrate diet, with low protein intake, were able to retain very considerable quantities of the N of ammonia (NH_4Cl). With larger quantities of ammonium citrate, they were able to maintain nitrogenous equilibrium for a period of fifteen days. They did not notice any elimination of retained nitrogen during the after periods. Grafe and Schläpfer, therefore, reached the conclusion that the animal has the power of synthesizing protein from carbohydrate and ammonium. Abderhalden's method was similar to Grafe's. His first dog received, during the fore-period of eight days, 40 grams of fat, 40 grams of sugar, 40 grams of starch and 5 grams of bone ash, per day. The nitrogen balance for that period, as was to be expected, was strongly negative (-1.83 to -2.95 per day). During the following three days, ammonium carbonate was added to the diet, and the nitrogen balance was -0.23 , -0.08 , -0.09 gram per day. During the after period, the nitrogen elimination was not any higher than during the fore-period. Experiments with ammonium acetate did not show such marked retention, but showed a very considerable retention. Abderhalden hesitatingly reached the conclusion that "the addition of ammonium salts as the only source of nitrogen to a diet consisting of carbohydrates and fats, exercises a distinct influence on the protein metabolism, by causing nitrogen retention." The relationship of this retention to the protein anabolism, he left an open question, suggesting, however, the probability that a reversible reaction had set in.

In later work on the subject,⁵ Abderhalden tends to incline more to the retention hypothesis, denying the possibility of the synthesis of protein from ammonia and carbohydrates. He writes: "It is no doubt possible in some cases to reduce the nitrogen elimination by ammonium salts. We have not observed any

⁴ E. Abderhalden: Fütterungsversuche mit vollständig bis zu Aminosäuren abgebautem Eiweiss und mit Ammonsalzen., *Zeitschr. f. physiol. Chem.*, lxxviii, p. 1, 1912.

⁵ E. Abderhalden and Paul Hirsch: Fortgesetzte Untersuchungen über die synthetischen Fähigkeiten der tierischen Zelle. Versuche über die Verwertung verschiedener Stickstoffquellen im Organismus des Hundes, *Zeitschr. f. physiol. Chem.*, lxxxii, p. 1, 1912.

prolonged positive nitrogen balance. Only in a few cases was it temporarily positive. It was, however, more negative in the after period. We are inclined to assume that the established nitrogen does not stand in any direct relation to the synthesis of protein. We have no reason for the assumption that the animal organism has the power of producing the different complexes for the variously built amino-acids from carbohydrates, and that the administered ammonia is used for their aminization. Such an assumption stands in contradiction to all the present conceptions of protein metabolism."

On examining Abderhalden's figures, one fails to notice any marked "Ausschwemmung" of the retained nitrogen in the after periods.

The experiments reported below were performed with the object of throwing light on several questions which presented themselves to us on reviewing the subject.

1. *Does the ammonia combine with some rest of the carbohydrate molecule in its retention; is the carbohydrate really necessary for the demonstration of the utilization of ammonia in the protein metabolism; and does the carbohydrate give rise to the non-nitrogenous fraction of the various amino-acids?*

Grafe maintains throughout his studies that large quantities of carbohydrates are necessary to get a satisfactory demonstration of the utilization of ammonia, and Abderhalden has accordingly kept his animals on a high caloric intake. The results of Grafe's and Abderhalden's experiments are very similar. The differences are only in the degree of retention. Grafe maintains that the differences in their results are to be sought in the difference of the carbohydrate supply, and that the quantity of the carbohydrate fed is the determining factor.

It seems to us that the understanding of the state of retention of the nitrogen involves this question directly. Is the carbohydrate a very important factor? Does the circulation of an excess of carbohydrate molecules in the blood bring about the synthesis with the ammonia to amino-acids, which either build up or spare body protein; or does the carbohydrate play a secondary rôle, and the ammonia the primary rôle?

We have attempted to solve this problem by feeding ammonium carbonate to starving dogs. If the carbohydrate is of importance,

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and if the ammonia in its retention is tied up with the carbohydrate molecule, the results should be negative. That is, none of the ammonia nitrogen should be retained. On the other hand, if on giving ammonium carbonate, a retention of the nitrogen should occur, Grafe's "carbohydrate" theory can safely be eliminated.

Dog 1 was permitted to fast for three days prior to the commencement of the experiment. Water was given *ad libitum*. The animal was kept in a metabolism cage, and the urine collected quantitatively. The periods of twelve hours were separated by catheterizing and washing the bladder with distilled water. The nitrogen was determined according to Kjeldahl, the ammonia according to Folin. The ammonium carbonate was prepared by Baker, and has the following composition: $(\text{NH}_4)_2\text{CO}_3 \cdot \text{NH}_4\text{CO}_2 \cdot \text{NH}_3$.

EXPERIMENT I.

Twelve-hour periods.

PERIOD	WEIGHT	TOTAL NITROGEN	NH ₄ N	NITROGEN BALANCE	REMARKS
I	8.32	1.01	0.058	-1.01	{ 1 gram of N in the form of ammonium carbonate given <i>per os</i> .
II		0.88	0.065	-0.88	
III		0.89	0.052	-0.89	
IV	8.05	1.28	0.076	-0.28	
V		0.87	0.054	-0.87	

This experiment shows very clearly that a good part of the nitrogen (more than half a gram) was retained, and was not eliminated in the after period.

To avoid the criticism of too short periods, the periods of the next experiments were extended to twenty-four hours.

EXPERIMENT II.

Twenty-four-hour periods.

PERIOD	WEIGHT	TOTAL NITROGEN	NH ₄ N	NITROGEN BALANCE	REMARKS
I	17.68	3.26	0.24	-3.26	{ Sixth fasting day. 2 grams of N in the form of ammonium carbonate given <i>per os</i> .
II		4.14	0.236	-2.14	
III	17.32	3.22	0.408	-3.22	

The results of this experiment corroborate the findings of the first. Both agree in showing that a considerable part of the nitrogen of the ammonia is retained, and that it is not eliminated in the after period.

EXPERIMENT III.

Twenty-four-hour periods.

PERIOD	WEIGHT	TOTAL NITROGEN	NITROGEN BALANCE	REMARKS
I		2.43	-2.43	Eighth fasting day.
II		2.32	-2.32	
III		2.39	-2.39	
IV		2.21	-2.21	
V	8.95	2.13	-2.13	
VI	8.85	1.96	-1.96	
VII	8.79	2.00	-2.00	
VIII	8.69	2.45	-2.45	
IX	8.74	2.16	-2.16	
X	8.66	3.12	-0.82	73 grams of meat = 2.3 grams of N.
XI	8.58	1.90	-1.90	
XII	8.48	1.75	-1.75	
XIII	8.44	2.13	+0.17	2.3 grams of N in the form of meat.
XIV	8.36	1.81	-1.81	
XV	8.20	1.62	-1.62	
XVI		2.74	-0.44	{ 2.3 grams of N in the form of ammonium carbonate <i>per os</i> .
XVII		1.83	-1.83	
XVIII	7.80	1.72	-1.72	{ 2.3 grams of N in form of ammonium carbonate given <i>subcutaneously</i> .
XIX		3.40	-1.10	
XX		2.91	-2.91	
XXI		2.37	-2.37	
XXII	7.48	3.97	-1.67	{ 2.3 grams of N in the form of urea given <i>per os</i> .
XXIII	7.32	2.00	-2.00	
XXIV		1.76	-1.76	
XXV	6.98	4.02	-1.72	2.3 grams of N in the form of urea.
XXVI	6.95	2.16	-2.16	

This experiment, in addition to the fact that it corroborates the first two, also shows that the nitrogen of ammonia may affect the nitrogen balance almost to the same extent as does the feeding of meat. Ammonia administered subcutaneously is eliminated quantitatively. Urea administered *per os* is eliminated quantitatively.

CHAPTER II. IRRIGATION

The first question that arises in the mind of the farmer is, "What is the best method of irrigation?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water." The next question is, "What is the best method of distributing the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water." The third question is, "What is the best method of controlling the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The fourth question is, "What is the best method of measuring the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The fifth question is, "What is the best method of maintaining the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The sixth question is, "What is the best method of disposing of the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The seventh question is, "What is the best method of utilizing the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The eighth question is, "What is the best method of storing the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The ninth question is, "What is the best method of distributing the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The tenth question is, "What is the best method of controlling the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The eleventh question is, "What is the best method of measuring the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The twelfth question is, "What is the best method of maintaining the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The thirteenth question is, "What is the best method of disposing of the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The fourteenth question is, "What is the best method of utilizing the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

The fifteenth question is, "What is the best method of storing the water?" The answer to this question is, "It depends upon the nature of the soil, the amount of water available, and the cost of the water."

unnecessary, and which makes the interpretation of their results considerably more difficult. Grafe's theory that the carbohydrates pass into union with the nitrogen of the ammonia is here proven to be erroneous. The most probable answer to the above questions, and the one most strongly in harmony with well-known physiological and chemical facts, is the following: the ammonia, because of its high concentration in the tissues, most probably in the intestinal wall and liver, reverses the process of deamination. By catabolism of protein in the cells, we understand a cleavage of protein into the constituent amino-acids that make up the protein molecule. These amino-acids are believed to be deaminized, the amino radical going into urea, and the non-nitrogenous fraction either broken down and burnt directly or utilized in the synthesis of glucose, glycogen and fat. It is at the point of this deamination that we believe the ammonia to exert its influence. It is now well established that the non-nitrogenous part of the deaminized amino-acids are either α -hydroxy or α -ketonic acids, both of which when administered into the body or perfused through the liver have the power of combining with the amino radical.

We have attempted to test this theory in the following way: Grafe, Abderhalden and the writers, in the experiments reported above, administered all the ammonium carbonate in a rather concentrated form. This causes the ammonia to be absorbed rapidly, and to exert a high mass action. It was thought that, by giving the ammonia in very high dilution and in small quantities at a time, the mass action might be eliminated.

The subject of this experiment was a man of about 70 kilos body weight, in medium flesh. The diet consisted of 200 grams of starch, 200 grams of cane sugar, 5 grams of table salt and 3000 cc. of water. The starch was the best of several preparations; the one used contained only 0.065 gram of nitrogen in the day's ration. The diet could fairly be termed nitrogen-free. It contained 1600 calories, not enough to place the individual in caloric equilibrium, but enough to spare body protein effectively. The water was consumed with the food, three times daily. The subject was placed on this diet for a fore-period of five days. The urine and feces were collected only during the last three days of the fore-period. The urine was analyzed for total nitrogen by the method of Kjeldahl, ammonia and creatinine by the methods of Folin, urea

Data in Metabolism

...the purine bodies by success-
 ...the copper bisulphate, the
 ...nitrogen content by the
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 ...was administered in
 ...the salt being dissolved
 ...The ingestion for the
 ...2.8 grams of nitro-
 ...ammonium carbonate
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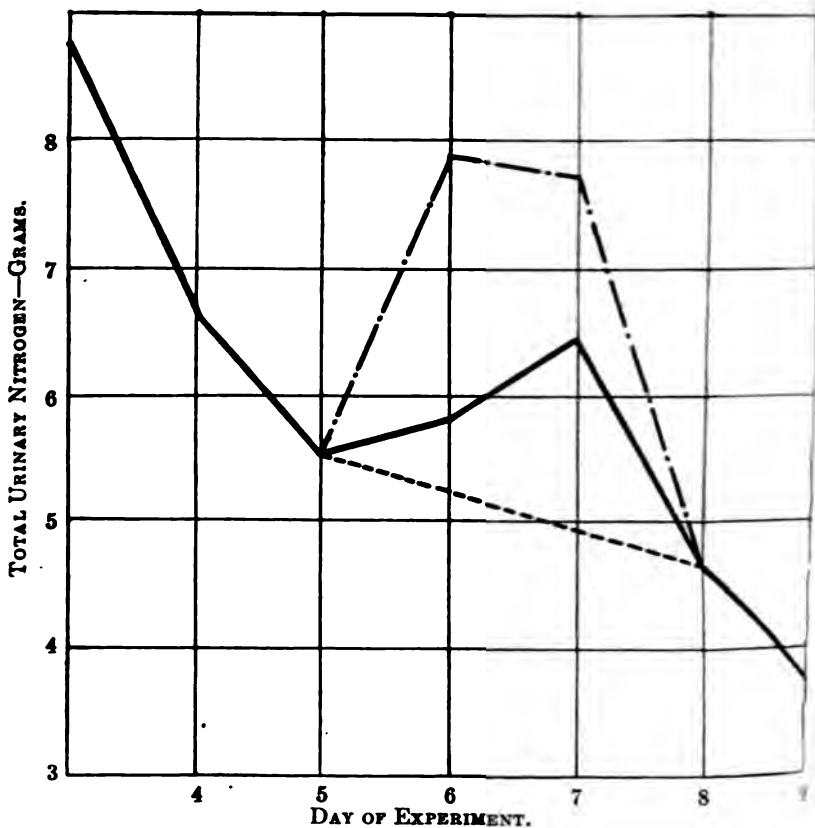
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The average fecal nitrogen, 0.76 per day, cannot be considered in any way above the normal. Obviously, the ammonium carbonate was fully resorbed. And the diarrhea made apparently no difference in this resorption. If there was any effect, there was possibly an increase in the secretory nitrogen eliminated, though, as stated, 0.76 gram of nitrogen per day cannot be considered high; it is within the usual limits for normal figures.

Strikingly constant are the figures for creatinine and purine and, with the exception of one day, of ammonia also. There is a short rise in the purine on one day, the second day of the test, leading possibly to the inference of increased nuclear catabolism in the liver, as the result of, or associated with, the presence of the excess of ammonium carbonate in the liver.

From an inspection of the figures for the total urinary nitrogen and urea, it is clear that there was a rise, associated with the ingestion of the ammonium carbonate, but which was not at all commensurate with the input. The greatest part of the administered ammonia was retained in the system, and was not eliminated in the after period. This shows that the ammonia is retained even when given in dilute form. It does not, however, vitiate the theory of the reversible action of ammonia on deamination. For dilute as the ammonia was when administered, we have no means of telling at what rate and in what concentration it was absorbed. We hope to test this again by dividing the ammonia into very small doses, administered at very short intervals during the day.

The curve yields a graphic reproduction of the data. The heavy continuous line represents the actual figures for urinary nitrogen. The dotted curve for the two days indicates approximately what would have been the curve of elimination had no ammonium carbonate been administered. The dashed line indicates what would have been the curve of elimination had all the nitrogen of the ingested ammonia been eliminated. It is clear that the larger fraction of the nitrogen has been retained.



SUMMARY.

Three experiments were performed on starving dogs, and the nitrogen output studied. Ammonium carbonate was given *per os*, and it was found that a considerable part of the nitrogen was retained and failed to be eliminated in the after period. When given subcutaneously, it was promptly eliminated.

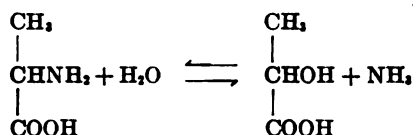
The administration of urea was followed by a complete elimination of all the nitrogen.

Ammonia administered to diabetic dogs was also retained, but to a larger extent than in normal dogs.

Ammonia given to man on a protein-free diet (0.065 gram per day) was retained to the extent of about two-thirds.

These experiments corroborate the findings of Grafe and Abderhalden, and also show that the presence of carbohydrates is not an obligatory factor in the retention of nitrogen from ammonia.

It is suggested that the ammonia nitrogen is retained because of a reversed reaction that leads to combination with the α -ketonic or α -hydroxy-acids to form amino-acids which may be used in the upbuilding or sparing of body protein. This may be illustrated in the simple reaction for the deamination of alanine.



Like every reaction, this reaction must be reversible under appropriate conditions. What the station of equilibrium in the animal body may be, we have no way of knowing. But according to the interpretation of the retention that we are inclined to accept, with the administration of large amounts of ammonia this reaction is reversed, probably in the liver. Whether such a situation as this might arise outside of the experiment, normally or pathologically—the formation of such amounts of ammonia as to reverse the reaction—is problematical. It is possible that such concentration of ammonia within the portal system could never occur naturally, in health or disease. In that event, this experiment would simply demonstrate the possibility of reversion of reaction in the animal body and add another illustration to the long list of demonstrations of the validity of physico-chemical laws within living bodies. We have used alanine as the simplest illustration of the reaction; and in view of the omnipresence of lactic acid within the organism, it is possible that the retention occurs largely or entirely in this state. The body could then make use of this alanine to the same extent and in the same way that alanine derived from the hydrolysis of protein is utilized.

If one does not incline to the view that the ammonia is retained in combination, one must assume that it is retained either in the state of ammonia or urea. The latter appears unlikely, in view of the prompt and complete elimination of urea in the direct experiment. The retention of ammonia as such, in the form of a salt, appears to us quite unlikely.

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STUDIES IN THE PURINE METABOLISM.

I. ON URICOLYSIS IN THE HUMAN SUBJECT.

BY ALONZO ENGLEBERT TAYLOR AND WILLIAM C. ROSE.

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(Received for publication, April 2, 1913.)

The purine bases are, according to almost universal belief, converted into uric acid or eliminated unchanged, this applying to both endogenous and exogenous purine bases. To what extent the elimination of uric acid, however, is to be regarded as expressive of the formation of uric acid in catabolism (the factor of circulatory retention being disregarded) is, despite much investigation, not yet clear. The most current opinion runs to the effect that a relatively large amount of the uric acid formed in metabolism, or ingested, is converted into urea under the influence of uricolytic ferments, such as have been isolated from the organs of lower animals. Without at this time entering into a discussion of the literature, we wish to report a carefully controlled experiment on the human subject. Experiments to be reported in a subsequent paper deal with the influence of the input of nitrogen and of glandular work upon the elimination of uric acid.

The subject of the experiment was a healthy young adult, a student of medicine, weighing about 60 kilos. The test extended over fourteen days. Wishing to exclude rigidly any possible influence of the input of nitrogen and of glandular work, the diet was constant in nitrogen and in calories during the entire period. The plan of the test was to place the subject for three days upon a diet of milk, egg, starch and sugar. During the following three days, a fraction of the milk-egg was substituted by sweetbreads containing the same amount of nitrogen. During the next four days this substitution was doubled. During the last four days the subject was again placed upon the diet of the first period. The nitrogen during the four periods was exactly 10 grams per

day. The caloric value of the diet was 2000 calories per day. The diet of the first period was prepared daily as follows. Four eggs were beaten into two liters of milk, and the nitrogen determined by the method of Kjeldahl. An amount of this mixture containing 10 grams of nitrogen was then measured off, and enough sugar and nitrogen-free starch added to bring the caloric value to the determined plane. The food was then cooked in the form of a custard, and ingested in three meals. This continued as stated for three days. Upon the fourth day, an amount of the mixture of milk and egg corresponding to 3 grams of nitrogen was withdrawn, and replaced by a portion of sweetbreads containing 3 grams of nitrogen. The sweetbreads were ground in a fine mill, and cooked in the form of a loaf, in which state duplicating estimations of nitrogen could be secured. The caloric input remained unchanged. After four days of this regimen, 3 further grams of the milk-egg nitrogen input were substituted by an equal amount of nitrogen in the form of the sweetbreads, the caloric input being unchanged. During the last four days the subject was returned to the diet of 10 grams of nitrogen in the form of milk and egg. Thus the nitrogen input of the tests was as follows:

	grams
<i>First period:</i> milk-egg nitrogen.....	10
<i>Second period:</i> milk-egg nitrogen, 7 grams; sweetbread nitrogen	3
<i>Third period:</i> milk-egg nitrogen, 4 grams; sweetbread nitrogen	5
<i>Fourth period:</i> milk-egg nitrogen.....	10

It was assumed that the digestion and assimilation of 1 gram of sweetbread nitrogen corresponded with that of 1 gram of milk-egg nitrogen. In the first and fourth periods the subject was on a purine-free diet; in the second and third periods, the subject was upon a known and controlled input of exogenous purine in the shape of nucleic acids of the sweetbreads. The feces were marked and collected. The urine was collected in periods, not in days, in order to avoid the fluctuations of daily eliminations. The figures in the tables represent therefore the daily averages of each period; and the evenness of these figures is of course due to this procedure. The urine was analyzed for total nitrogen by the method of Kjeldahl; for urea by the method of Benedict; for ammonia and creatinine by the methods of Folin; for uric acid by

the new colorimetric method of Folin; the dried feces by the method of Kjeldahl. The purine bases were estimated as follows.

Six hundred cc. of urine are freed of phosphates by the addition of 50 cc. of magnesia mixture, 50 cc. of 5 per cent ammonia added, filtered and of the filtrate 600 cc. taken for the estimation of purine. The purine bodies are first precipitated according to the method of Salkowski with silver nitrate. The precipitate is collected, washed free of chlorides, suspended in water, a drop of hydrochloric acid added, the silver precipitated with hydrogen sulphide, heated, filtered hot and the filtrate evaporated to dryness at about 70°. Important in the operation is a clear filtrate after precipitation of silver sulphide, quick filtration of the hot solution, and certainty that at all times the reaction of this filtrate is acid. The dried residue is then taken up in hot water, the smallest traces of sodium carbonate added until the uric acid passes entirely into solution, the hot solution faintly acidulated with acetic acid and filtered at once into a marked 250 cc. flask, the filter paper washed with hot water, the flask filled to the mark with hot water, and then two portions of 100 cc. each measured into beakers, and the purines precipitated with copper sulphate and sodium bisulphite according to the method of Krüger. The washed precipitates are finally transferred to Kjeldahl flasks and the nitrogen estimated. To determine the purine nitrogen of the total urine the obvious calculations must be carried out. To determine the nitrogen of the purine bases, the nitrogen of the uric acid determined apart must be subtracted. This method has been applied to urines to which known additions of purine bases and of uric acid have been made, with checking results. The new Folin method for the estimation of uric acid has been found to check well with the results of the method of Salkowski, and is carried out in a twentieth of the time.

The following table presents, in terms of nitrogen daily, the results of the experiment. All figures are rounded.

DAYS.....	1, 2, 3	4, 5, 6	7, 8, 9, 10	11, 12, 13, 14
Total urinary N.....	8.9	8.7	9.1	8.8
Urea N (+ NH ₃).....	7.3	7.1	7.1	7.05
Creatinine N.....	0.58	0.55	0.56	0.47
Purine N.....	0.11	0.17	0.26	0.10
Uric acid N.....	0.09	0.14	0.24	0.07
Rest N.....	0.91	0.88	1.18	1.18
N input.....	10.0	10.0	10.0	10.0
Purine N input.....	0	0.17	0.34	0
Fecal N output, Average.....	0.5	0.5	0.5	0.5

The figure given for the purine nitrogen of the sweetbreads is approximate only; it is certainly too low. The nucleic acids were freed according to Neumann, precipitated with alcohol, then hydrolyzed with hydrochloric acid, then estimated just as described for purine nitrogen in the urine. The stools contained but mere traces of nucleic acid in the cell- and bacteria-free filtrate; apparently, judging by this and by the nitrogen of the stools, the digestion of the sweetbreads was normal and complete.

From these figures it is clear that although the replacement of milk-egg nitrogen by sweetbread nitrogen results in a rise in the purine nitrogen of the urine, an expression of the elimination of purines derived from the catabolism of the nucleic acids of the sweetbreads, this increment in elimination is less than half of the known input of purine in the state of nucleic acids in the sweetbreads. The proportional rise in the elimination during the third period as contrasted with the figure for the second period, must have been purely accidental. It is clear from these figures that the larger portion of the ingested purine was either destroyed in the alimentary tract prior to resorption, or was converted in the metabolism into non-purine (presumably into urea); since less than half of the ingested amount was recovered in the urine. It is also clear from the figures that the ingestion of a moderate amount of purine bases does not lead to increase in the purine bases in the urine, but solely to increase in uric acid.

ON GLYOXALASE.

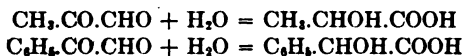
By H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York.)

(Received for publication, April 2, 1913.)

In a recent communication¹ the writers have given a preliminary account of the formation of α -hydroxy-acids from α -ketonic aldehydes as the result of ferment action. The present paper is concerned with a more detailed study of the reaction.

The catalyst with which we are concerned effects the rapid conversion of methyl glyoxal into lactic acid and of phenyl glyoxal into mandelic acid—a reaction which is readily imitated *in vitro* by the action of alkalies.



It is probable that other glyoxals may undergo similar changes but, from a biochemical standpoint, the formation of lactic acid from methyl glyoxal is of particular interest on account of the relation of the latter substance to the carbohydrates.

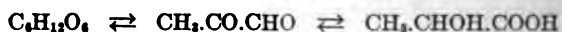
We shall present evidence indicating the enzyme character of the catalyst in question and, in conformity with current nomenclature, we propose that it be named "glyoxalase."

We find that a solution exhibiting strong catalytic activity, such as may be made by straining an aqueous extract of muscle or liver from an exsanguinated dog or rabbit, may be heated for a short time to 48° without much injury but at once loses its activity on heating to 60°. On allowing the tissue extract to stand at room temperature, without addition of antiseptics, the activity is lost relatively slowly. After forty-eight hours there is not much diminution in activity, but after four or five days the solution contains but little of the enzyme.

¹ This *Journal*, xiv, p. 155, 1913.

Experiments aiming at a rough purification of the catalyst by precipitating filtered tissue extracts with alcohol were unsuccessful, but by precipitating with solid ammonium sulphate and dialyzing the suspension of the precipitate in water, an active solution of the enzyme was obtained.

In order to obtain some information as to the conditions under which the enzyme is active, we have made experiments as to the effect of the presence of acid and alkali. We find that, above a certain small concentration, acids such as acetic acid (0.1 per cent) exert not only an inhibitory effect, but actually destroy the enzyme so that no action is observable on subsequent neutralization. Moderate amounts of alkali (0.1 per cent Na_2CO_3), on the other hand, have no such action. The inhibitory effect of acids would seem to be of importance, for it obviously indicates a regulatory mechanism for the action of the enzyme; for both methyl glyoxal and the glucose which may yield it are substantially neutral, while an accumulation of the product of the enzyme action, lactic acid, would automatically inhibit further acid formation. It appears likely that we have to do with a delicately adjusted equilibrium which may roughly be represented as follows:



Additional support as to the possibility of some such series of changes occurring in the animal body is furnished by the fact that we have recently been able to demonstrate the reversibility of the methyl glyoxal lactic acid transformation by experiments *in vitro*. In addition, by the action of sodium phosphate upon glucose, we have been able to obtain a substance which yields hydrazine derivatives of methyl glyoxal and which is therefore either methyl glyoxal or some closely related substance. These experiments, and others upon the fate of methyl glyoxal in the diabetic organism, will be reported in future communications.

The fact that acid, whether formed by the action of the enzyme or added to the solution, inhibits the reaction, makes it necessary to provide for the automatic neutralization of the solution when observing the action of the enzyme. Indeed the speed of lactic acid formation is so great when moderate amounts of tissue extract are employed that it depends substantially upon the rate of neutralization. If neutral methyl glyoxal solution is added to a neutral

tissue extract and the mixture incubated at 37°, it is noticed that after a few minutes the solution becomes acid and a heavy precipitate of proteins is formed. The acidity may increase until it is about equal to a $\frac{N}{100}$ solution of lactic acid (0.09 per cent) and then no further formation of acid occurs. For purposes of neutralization we have employed sodium bicarbonate and sodium phosphate and freshly precipitated calcium carbonate. The latter appears to be preferable. The disadvantage of the phosphate is that such large amounts are necessary to neutralize relatively small quantities of lactic acid and, while the action of the carbonate is slower, it has little or no action upon the glyoxals and it is not possible for excessive alkalinity to develop.

One of the most curious facts connected with glyoxalase is that its action on both methyl glyoxal and phenyl glyoxal does not yield a single optically active form of the corresponding hydroxy-acid, but a mixture of both forms in unequal proportions. Thus methyl and phenyl glyoxal give a mixture of laevo and inactive lactic and mandelic acids. At first we were inclined to ascribe the formation of the inactive acids to hydrolysis taking place independently of the enzyme, but further investigation does not bear out this supposition, and at present we are inclined to the belief that both dextro and laevo acids are formed as the result of enzyme activity. The evidence for this is based upon the following facts: Firstly: Both forms are produced under conditions unfavorable to hydrolysis except by enzyme action, *e.g.*, in faintly acid solution. Secondly: The acids obtained show variations in the proportions of laevo and dextro forms. In fact, in the case of one experiment, an enzyme solution which at first gave chiefly laevo mandelic acid when acting on phenyl glyoxal, on standing and subsequently acting on fresh phenyl glyoxal, gave mandelic acid containing an excess of the dextro acid. This effect was certainly not due to bacterial action, since on further standing the enzyme was destroyed and no appreciable amount of mandelic acid could be obtained.

Judging by analogy, it seems unlikely that a single enzyme would effect an incomplete asymmetric synthesis, such as the conversion of methyl and phenyl glyoxals into mixtures of the laevo and dextro lactic and mandelic acids. So far as the facts are at present available, it would seem probable that more than one enzyme is concerned in the observed reactions.

It is certainly surprising that glyoxalase when acting upon methyl glyoxal should produce an excess of the laevo lactic acid, whereas the dextro acid is the variety ordinarily found in animal tissues. Levene and Meyer² have recently shown that dextro lactic acid is exclusively produced by the action of leucocytes upon glucose and, simultaneously with ourselves, revived Nef's view that methyl glyoxal is an intermediate step in the conversion of glucose into lactic acid. What rôle, if any, laevo lactic acid plays in animal metabolism must be the subject of future investigation, for it is clear that the stereochemical relations are by no means simple. It may be noted, however, that the occurrence of inactive lactic acid in animal tissues has, in the past, been asserted, although Moriya³ believed on the basis of rather inadequate experiments that these statements were inaccurate.

An idea of the striking activity of glyoxalase may be gained from the fact that 100 cc. of an extract prepared by shaking liver tissue with five parts of saline, containing less than a gram of organic matter, is able to convert more than 2 grams of methyl glyoxal into lactic acid in less than fifteen minutes if provision is made for the prompt neutralization of the acid as formed.¹⁷

It would seem likely that glyoxalase may have a wide distribution, and we have already observed its presence in yeast and in the oyster. Certain lactic acid bacilli which were tested, gave a negative result possibly on account of the failure of the enzyme, if present, to leave the intact cells. We find that the action of glyoxalase observed in blood is confined entirely to the cells, the serum being inactive.

EXPERIMENTAL.

Preparation of glyoxalase extract from animal tissues.

The animal (dog or rabbit) was bled and washed out with physiological saline under ether anaesthesia. Immediately after death the tissues to be used were removed, finely minced and stirred up with warm (35°C.) saline for a few minutes. To each 100 cc. of saline were added 20 grams of minced tissue. The liquid was then strained through muslin. In most experiments the turbid extract

² This *Journal*, xiv, p. 149, 1913.

³ *Zeitschr. f. physiol. Chem.*, xliii, p. 397.

was used directly, but it may be filtered through paper without losing its activity. The filtrate may also be precipitated with solid ammonium sulphate and on dissolving the precipitate in water and dialyzing an active solution of the enzyme may be obtained.

Investigation of the action of glyoxalase extract on phenyl and methyl glyoxal.

a. Phenyl glyoxal. To a known volume of extract was added a solution of phenyl glyoxal and the substance used for controlling the acidity of the mixture—sodium phosphate, sodium carbonate or chalk. After incubation for several hours at 37°C. in the presence of a small amount of toluene solid ammonium sulphate—roughly 50 grams to 100 cc.—was added and the liquid was heated in a water bath for ten to fifteen minutes. The ammonium sulphate precipitates any unchanged phenyl glyoxal. The mixture was then allowed to cool, acidified with a few cubic centimeters of phosphoric acid, and, after effervescence had subsided, filtered off from precipitated protein which was washed with ammonium sulphate solution. The filtrate, to which a further small quantity of phosphoric acid was added, was then extracted three times with small amounts of ether, each extract being washed with about 5 cc. of water. The residue, after evaporation of the ether, was dissolved in water (10 cc.), filtered, examined polarimetrically in a 2 dm. tube, and subsequently its acidity determined by titration of 5 cc. of the solution with decinormal sodium hydrate.

b. Methyl glyoxal. In this case the incubation mixture was prepared as for phenyl glyoxal, but for the extraction of the lactic acid produced the following method was first adopted. The protein was precipitated with ammonium sulphate and filtered off as before, and the filtrate, acidified with phosphoric acid, was extracted with ether for many hours. The residue from the ether extract was taken up in water, examined in the polarimeter, and an aliquot part was titrated with decinormal alkali to determine the acidity. The remainder was converted into the zinc salt for identification and analysis.

In later experiments the incubated mixture was faintly acidified with acetic acid and then evaporated nearly to dryness on the water

bath. The protein which had separated out was filtered off and washed several times with warm water. The filtrate was taken nearly to dryness again, acidified with phosphoric acid and ground up with gypsum, sufficient of the latter being used to give a dry powder, and then treated with ether in a Soxhlet apparatus. This was found to give a more complete extraction than the former method.

I. Effect of temperature on glyoxalase.

a. 100 cc. 20 per cent extract (liver and skeletal muscle of rabbit) + 0.2 gram phenyl glyoxal hydrate in 6 cc. water, incubated 16 hours at 37°C in presence of toluene. Rotation of mandelic acid solution in 2 dm. tube, -1.67° .

b. As in a, except that the 20 per cent extract was first heated up to 47°C. Rotation of mandelic acid solution in 2 dm. tube, -1.52° .

c. As in a, except that extract was previously heated to 60°C. Rotation of mandelic acid solution in 2 dm. tube, -0.08° . Acidity = 0.4 cc.

Glyoxalase is evidently destroyed at some temperature above 48° but below 60°.

II. Influence of acid and alkali on glyoxalase.

a. To 100 cc. 20 per cent extract (liver and skeletal muscle of rabbit) was added 0.1 cc. glacial acetic acid. After incubating for 3½ hours, 5 cc. of chalk suspended in water and 0.2 gram phenyl glyoxal in 5 cc. of water were added. Incubated for 18 hours at 37°C. Rotation of mandelic acid solution (10 cc.) in 2 dm. tube, $+0.03^{\circ}$. The acidity was less than 1 cc. and was in part due to acetic acid.

b. Similar to a but no chalk added and phenyl glyoxal added at once. Rotation of mandelic acid solution, -0.25° .

c. A mixture containing 100 cc. 20 per cent extract (liver and skeletal muscle of rabbit), 0.2 gram phenyl glyoxal and 0.11 gram sodium carbonate was incubated as usual. After digestion the reaction liquid was distinctly acid to litmus. Rotation of mandelic acid solution (10 cc.) in 2 dm. tube, -2.07° .

The above experiments show that the action of glyoxalase is almost completely inhibited by 0.1 per cent acetic acid and that this concentration of acid completely destroys the enzyme in less than three and one half hours so that no activity is observed on neutralization. No inhibitory effect is observed with 0.1 per cent sodium carbonate.

III. Changes observed in the enzyme solution on standing.

The glyoxalase extract (20 per cent, liver and skeletal muscle of rabbit) stood at room temperature, without antiseptics, but before using was treated with toluene.

a. 5 hours after preparation. 50 cc. extract + 5 cc. chalk suspension + 0.2 gram phenyl glyoxal hydrate in 5 cc. of water + toluene: incubated for 18 hours at 37°C. Rotation of mandelic acid solution (10 cc.) in 2 dm. tube, $-2.33^\circ = 0.074$ gram *l*-mandelic acid. Acidity equaled 0.1216 gram total mandelic acid.

A similar experiment, using only 25 cc. of extract gave an observed rotation of -1.60° , equivalent to 0.051 mandelic acid, and an acidity corresponding to 0.086 gram total mandelic acid.

b. 24 hours after preparation. 100 cc. extract + 5 cc. chalk suspension + 0.2 gram phenyl glyoxal in 5 cc. water + toluene: incubated for 18 hours at 37°C. Rotation of mandelic acid solution (10 cc.) in 2 dm. tube, $-2.42^\circ = 0.077$ gram *l*-mandelic acid. Acidity = 0.1216 gram total mandelic acid.

c. 48 hours after preparation. Incubation mixture and time as in b. Rotation of mandelic acid solution (10 cc.) in 2 dm. tube, $-1.75^\circ = 0.056$ gram *l*-mandelic acid. Acidity = 0.1034 gram total mandelic acid.

d. 72 hours after preparation. Experiment made as in b. Rotation of mandelic acid solution (10 cc.) in 2 dm. tube, $+0.27^\circ = 0.0086$ gram *d*-mandelic acid. Acidity = 0.0669 gram total mandelic acid.

e. 120 hours after preparation. Experiment made as in b. Rotation of mandelic acid solution (10 cc.) in 2 dm. tube, 0° . Acidity, 1.0 cc. The enzyme was obviously destroyed.

It will be noted that in experiment *d*, dextro mandelic acid was obtained while in all the previous ones the laevo acid was chiefly formed, although the diminution of the laevo form was evident in *c*.

IV. Formation of lactic acid by the action of glyoxalase on methyl glyoxal.

a. 100 cc. 20 per cent extract (dog's liver) + 1 gram methyl glyoxal in 40 cc. water + 80 cc. 10 per cent sodium phosphate solution + toluene. The phosphate solution was added gradually as required to neutralize acidity.

b. 100 cc. 20 per cent extract (dog's liver) + toluene + 10 per cent sodium phosphate solution.

c. 100 cc. boiled 20 per cent extract (dog's liver) + 1 gram methyl glyoxal in 40 cc. water + toluene + 10 per cent sodium phosphate solution. The mixture *a* and controls *b* and *c* were incubated at 37°C. for 3 hours.

Lactic acid was extracted as described in a continuous extraction apparatus. The ether residues were taken up in 12 cc. of water examined polarimetrically in a 2.2 dm. tube, and 1 cc. subsequently titrated to determine the acidity.

- a. Observed rotation, -0.07° . 1 cc. required 5.8 cc. alkali equivalent to 0.6264 gram lactic acid.
- b. Observed rotation, 0° . 1 cc. required 0.1 cc. alkali.
- c. Observed rotation, $+0.02^{\circ}$. 1 cc. required 0.3 cc. alkali.
- d. 100 cc. 20 per cent extract (liver and skeletal muscles of rabbit) + 10 cc. chalk suspension + 1 gram methyl glyoxal in 40 cc. of water. Incubated 18 hours at 37°C .

Worked up lactic acid with gypsum as described and extracted in Soxhlet apparatus with ether.

Ether extract made up to 10 cc. Rotation in 2 dm. tube, -0.2° . 1 cc. required 10.1 cc. alkali = 0.909 gram lactic acid.

V. Inhibition of activity of enzyme due to the production of lactic acid.

100 cc. of 20 per cent extract (liver and skeletal muscles of rabbit) were mixed with 1 gram of methyl glyoxal in 40 cc. of water and incubated at 37°C . for 18 hours. The precipitate of protein produced was filtered off in the cold. 55 cc. of the solution were titrated with decinormal sodium hydrate and required 5.2 cc. This corresponds to a 0.085 per cent solution of lactic acid, and at this acidity glyoxalase ceases to convert methyl glyoxal into lactic acid.

The lactic acid obtained from different experiments was all worked up to sine lactate. This was recrystallized and analyzed.

Crop I: 0.2944 gram lost 0.0492 gram H_2O at 120°C = 16.7 per cent.

Crop II: 0.2749 gram lost 0.0386 gram H_2O at 120°C = 14.03 per cent.

0.2541 gram zinc lactate (anhydrous) gave on ignition 0.0853 gram ZnO = 33.56 per cent ZnO . Theory requires 33.4 per cent.

Crop I. 0.3052 gram of the recrystallized salt in 10 cc. aqueous solution gave a rotation of $+0.32^{\circ}$ in a 2 dm. tube.

$$[\alpha]_D = +6.36^{\circ}$$

Crop II. 0.2749 gram recrystallized salt in 10 cc. solution gave a rotation of $+0.42^{\circ}$.

$$[\alpha]_D = +8.89^{\circ}$$

Presence of glyoxalase in blood cells.

The serum and cells of defibrinated dog's blood were separated by means of the centrifuge. After the cells had been washed several times with saline, they were added to solutions containing chalk and phenyl glyoxal. In one series of experiments, 25 cc. of blood serum were added to a solution of 0.1 gram phenyl glyoxal in 25 cc. of water, and blood cells made up to 50 cc. with saline were added to a similar solution. These were incubated at 37°C . in the presence of toluene for twenty hours. The mandelic acid

was worked up as usual and the solutions (10 cc.) examined in a 2 dm. tube gave readings of 0° and -0.45° , respectively. Glyoxalase was evidently absent from the serum but present in the cells.

Using chalk to neutralize the acid formed, it has been possible to obtain large rotations, using the cells from only 5 cc. of blood.

Presence of glyoxalase in other organisms.

Yeast. A mixture of 5 grams of pressed yeast in 50 cc. of water with 0.2 gram phenyl glyoxal and 5 cc. of chalk suspension was incubated for 24 hours at 37°C . in presence of toluene. The mandelic acid was worked up in the usual manner and was readily obtained in crystalline form: the rotation of the solution (10 cc.) in a 2 dm. tube was -0.42° . Glyoxalase was evidently present.

Oyster. 25 grams of the minced tissues were added to a solution of 0.2 gram phenyl glyoxal in 50 cc. of water, 5 cc. of a chalk suspension and a few drops of toluene. The mixture was incubated for 24 hours at 37°C . and mandelic acid extracted in the usual manner. Rotation of solution (10 cc.), -0.42° . Glyoxalase was undoubtedly present.

Similar experiments to the above gave negative results when extracts of young potatoes or cultures of *B. bulgaricus* were employed.

STUDIES IN THE BLOOD RELATIONSHIP OF ANIMALS AS DISPLAYED IN THE COMPOSITION OF THE SERUM PROTEINS. II.

A COMPARISON OF THE SERA OF THE OX, SHEEP, HOG, GOAT, DOG, CAT AND GUINEA PIG WITH RESPECT TO THEIR CONTENT OF VARIOUS PROTEINS.

BY J. HOMER WOOLSEY.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, April 3, 1913.)

In the following investigations I have employed Robertson's refractometric method¹ of determining the concentrations of the various proteins contained in blood sera.

The animals employed were in some instances fasted for a definite period preceding the determinations; in the majority of instances normally fed animals were employed and their sera analyzed at an undetermined period after they had last fed. These animals are distinguished below as "normal" while the fasting animals are so designated. The sera were obtained by defibrinating the fresh blood, either by whipping or by shaking up with glass beads, and centrifuging the defibrinated blood. In each case, with the exceptions noted below, the reported analytical results are the average of four closely agreeing determinations made upon the same sample of serum.

A. Results obtained with ox serum.

The following were the results yielded by four different samples of ox serum obtained from "normal" animals at the time of slaughter in a neighboring meat company's plant.

¹ T. Brailsford Robertson: this *Journal*, xi, p. 179, 1912.

TABLE 1.
Ox serum.

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
1	0.52 \pm 0.04	1.94 \pm 0.15	5.57 \pm 0.2	7.51 \pm 0.2
2	0.59 \pm 0.04	2.30 \pm 0.15	5.27 \pm 0.2	7.44 \pm 0.2
3	0.62 \pm 0.04	2.34 \pm 0.15	5.18 \pm 0.2	7.52 \pm 0.2
4	0.73 \pm 0.04	2.26 \pm 0.15	5.28 \pm 0.2	7.54 \pm 0.2
Average	0.61 \pm 0.04	2.21 \pm 0.15	5.32 \pm 0.2	7.50 \pm 0.2

The figure following the \pm sign is the possible error in the determination due to a possible error of 1' in reading the angle of total reflection.

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, they yield the following figures.

"Insoluble" globulin.....	8.1	$\left\{ \begin{array}{l} 9.6 \\ 6.7 \end{array} \right\}$	(\pm 0.4)
Total globulins.....	29.0	$\left\{ \begin{array}{l} 31.0 \\ 25.0 \end{array} \right\}$	(\pm 2.0)
Total albumins.....	70.0	$\left\{ \begin{array}{l} 74.0 \\ 68.0 \end{array} \right\}$	(\pm 2.0)

The first figure opposite each group represents the average percentage; the upper figures immediately following, the highest percentage observed in any individual; the lower figure, the lowest percentage observed in any individual; and the figure in brackets, the plus or minus error in the estimation of these percentages which would be brought about by an error of 1' in reading the angle of total reflection.

Previous estimates of the proteins in ox serum are the following.

T. B. Robertson:² Determinations made upon the serum from one animal by the refractometric method.

"Insoluble" globulin.....	8.9 (\pm 0.4)
Total globulins.....	36.0 (\pm 2.0)
Total albumins.....	64.0 (\pm 2.0)

O. Hammarsten:³ Determinations made upon five animals; globulins precipitated by saturation of the serum with $MgSO_4$; the albumins determined by subtracting the globulins from the total proteins.

Total globulins.....	56	$\left\{ \begin{array}{l} 64 \\ 46 \end{array} \right\}$
Total albumins.....	45	$\left\{ \begin{array}{l} 56 \\ 36 \end{array} \right\}$

² T. Brailsford Robertson: *loc. cit.*

³ O. Hammarsten: *Arch. f. d. ges. Physiol.*, xvii, p. 461, 1878.

B. Results obtained with sheep serum.

The following were the results yielded by four different samples of sheep serum obtained from "normal" animals.

TABLE 2.
Sheep serum.

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
5	0.44 ± 0.04	1.10 ± 0.15	6.28 ± 0.2	7.38 ± 0.2
6	0.48 ± 0.04	1.25 ± 0.15	5.59 ± 0.2	6.64 ± 0.2
7	0.46 ± 0.04	1.07 ± 0.15	5.47 ± 0.2	6.55 ± 0.2
8	0.33 ± 0.04	1.17 ± 0.15	4.44 ± 0.2	5.61 ± 0.2
Average	0.42 ± 0.04	1.14 ± 0.15	5.44 ± 0.2	6.59 ± 0.2

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, they yield the following figures.

"Insoluble" globulin.....	6.4	$\left\{ \begin{array}{l} 7.0 \\ 5.9 \end{array} \right\}$	(± 0.4)
Total globulins.....	17.0	$\left\{ \begin{array}{l} 21.0 \\ 14.0 \end{array} \right\}$	(± 2.0)
Total albumins.....	82.0	$\left\{ \begin{array}{l} 85.0 \\ 78.0 \end{array} \right\}$	(± 2.0)

Previous estimates of the proteins in sheep serum are the following.

J. Lewinski:⁴ Globulins precipitated by saturation of the serum with $MgSO_4$; albumins determined by subtracting the globulins from the total proteins.

Total globulins.....	44
Total albumins.....	56

C. Results obtained with hog serum.

The following were the results yielded by four different samples of hog serum obtained from "normal" animals.

⁴ J. Lewinski: *Arch. f. d. ges. Physiol*, c, p. 611, 1903.

TABLE 3.
Hog serum.

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
9	0.37 \pm 0.04	3.17 \pm 0.15	4.01 \pm 0.2	7.19 \pm 0.2
10	0.48 \pm 0.04	1.98 \pm 0.15	5.21 \pm 0.2	7.19 \pm 0.2
11	0.48 \pm 0.04	3.34 \pm 0.15	4.96 \pm 0.2	8.50 \pm 0.2
12	0.51 \pm 0.04	2.47 \pm 0.15	5.25 \pm 0.2	7.72 \pm 0.2
Average	0.46 \pm 0.04	2.76 \pm 0.15	4.86 \pm 0.2	7.60 \pm 0.2

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, they yield the following figures.

"Insoluble" globulin.....	6.0	$\left\{ \begin{array}{l} 6.6 \\ 5.0 \end{array} \right\}$	(\pm 0.4)
Total globulins.....	36.0	$\left\{ \begin{array}{l} 44.0 \\ 28.0 \end{array} \right\}$	(\pm 2.0)
Total albumins.....	64.0	$\left\{ \begin{array}{l} 73.0 \\ 56.0 \end{array} \right\}$	(\pm 2.0)

Previous estimates of the proteins in hog serum are the following.

J. Lewinski:⁴ Globulins precipitated by saturation of the serum with MgSO₄; albumins determined by subtracting the globulins from the total proteins.

Total globulins.....	40
Total albumins.....	60

D. Results obtained with goat serum.

The following were the results yielded by two different samples of goat serum obtained from "normal" animals.

TABLE 4.
Goat serum.

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
13	0.38 \pm 0.04	1.41 \pm 0.15	5.03 \pm 0.2	6.44 \pm 0.2
14	0.54 \pm 0.04	1.72 \pm 0.15	5.59 \pm 0.2	7.31 \pm 0.2
Average	0.46 \pm 0.04	1.56 \pm 0.15	5.31 \pm 0.2	6.87 \pm 0.2

⁴ J. Lewinski; *loc. cit.*

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, they yield the following figures.

"Insoluble" globulin.....	6.5	$\left\{ \begin{array}{l} 7.4 \\ 5.5 \end{array} \right\}$	(± 0.4)
Total globulins.....	22.0	$\left\{ \begin{array}{l} 23.0 \\ 20.0 \end{array} \right\}$	(± 2.0)
Total albumins.....	75.0	$\left\{ \begin{array}{l} 76.0 \\ 73.0 \end{array} \right\}$	(± 2.0)

Previous estimates of the proteins in goat serum are the following.

C. Quinan:⁴ Determinations made upon one animal at intervals of one month in four series of analyses; "insoluble" globulin precipitated by CO_2 ; albumins determined by N determinations upon filtrate freed of globulins by MgSO_4 ; globulins determined by subtracting the albumins from the total proteins.

"Insoluble" globulin.....	9.4
Total globulins.....	46.0
Total albumins.....	54.0

E. Results obtained with dog serum.

The following were the results yielded by five different samples of dog serum obtained from animals fasted for forty-eight hours. In each case the reported analytical results are the average of three closely agreeing determinations upon the same sample of serum.

TABLE 5.
Dog serum.

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
15	0.38 ± 0.04	0.81 ± 0.15	5.66 ± 0.2	6.47 ± 0.2
16	0.57 ± 0.04	1.25 ± 0.15	6.24 ± 0.2	7.50 ± 0.2
17	0.59 ± 0.04	1.79 ± 0.15	4.98 ± 0.2	6.72 ± 0.2
18	0.67 ± 0.04	1.39 ± 0.15	6.69 ± 0.2	8.08 ± 0.2
19	0.55 ± 0.04	1.31 ± 0.15	5.24 ± 0.2	6.57 ± 0.2
Average	0.55 ± 0.04	1.31 ± 0.15	5.76 ± 0.2	7.07 ± 0.2

⁴C. Quinan: *Univ. of Calif. Publ. Path.*, i, p. 1, 1903.

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained.

"Insoluble" globulin.....	7.7	$\left\{ \begin{array}{l} 8.8 \\ 5.9 \end{array} \right\}$	(± 0.4)
Total globulins.....	18.0	$\left\{ \begin{array}{l} 27.0 \\ 12.0 \end{array} \right\}$	(± 2.0)
Total albumins.....	81.0	$\left\{ \begin{array}{l} 87.0 \\ 74.0 \end{array} \right\}$	(± 2.0)

Previous estimates of the proteins in dog serum are the following.

J. Lewinski:⁷ Globulins precipitated by saturation of the serum with $MgSO_4$; albumins determined by subtracting the globulins from the total proteins.

Total globulins.....	42
Total albumins.....	58

G. Salvioli:⁸ Globulins precipitated by saturation of the serum with $MgSO_4$; albumins by subtracting the globulins from the total proteins.

Total globulins.....	$37 \left\{ \begin{array}{l} 49 \\ 23 \end{array} \right\}$
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F. Results obtained with cat serum.

The following were the results yielded by four different samples of cat serum obtained from animals fasted for forty-eight hours. Two determinations were made upon each sample of serum.

TABLE 6.
Cat serum.

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
20	0.64 ± 0.04	4.09 ± 0.15	5.0 ± 0.2	9.09 ± 0.2
21	0.48 ± 0.04	2.09 ± 0.15	6.02 ± 0.2	8.11 ± 0.2
22	0.55 ± 0.04	2.03 ± 0.15	6.32 ± 0.2	8.35 ± 0.2
23	0.52 ± 0.04	2.05 ± 0.15	6.10 ± 0.2	8.15 ± 0.2
Average	0.55 ± 0.04	2.56 ± 0.15	5.86 ± 0.2	8.42 ± 0.2

⁷ J. Lewinski: *loc. cit.*

⁸ G. Salvioli: *Arch. f. (Anat. u.) Physiol.*, 1881, p. 269.

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained.

"Insoluble" globulin.....	6.5	$\left\{ \begin{array}{l} 7.1 \\ 5.9 \end{array} \right\}$	(± 0.4)
Total globulins.....	30.0	$\left\{ \begin{array}{l} 45.0 \\ 24.0 \end{array} \right\}$	(± 2.0)
Total albumins.....	69.0	$\left\{ \begin{array}{l} 76.0 \\ 55.0 \end{array} \right\}$	(± 2.0)

G. Results obtained with guinea pig serum.

The following were the results yielded by one sample of guinea pig serum obtained from ten animals fasted for twenty-four hours. Two determinations were made upon the sample of serum..

TABLE 7.
Guinea pig serum.

EXPERIMENT	"INSOLUBLE" GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
24	0.25 ± 0.04	0.93 ± 0.15	5.01 ± 0.2	5.94 ± 0.2

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained.

"Insoluble" globulin.....	4.2 (± 0.4)
Total globulins.....	16.0 (± 2.0)
Total albumins.....	84.0 (± 2.0)

SUMMARY.

The following table summarizes the average results obtained.

	PERCENTAGE OF THE TOTAL PROTEINS IN THE SERA OF						
	Ox	Sheep	Hog	Goat	Dog	Cat	Guinea Pig
"Insoluble" globulin	8.1	6.4	6.0	6.5	7.7	6.5	4.2
Total globulin.....	29.0	17.0	36.0	22.0	18.0	30.0	16.0
Total albumin.....	70.0	82.0	64.0	75.0	81.0	69.0	84.0

STUDIES ON THE THEORY OF DIABETES.

I. SARCOLACTIC ACID IN DIABETIC MUSCLE.

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The primary chemical disability in diabetes mellitus and pancreas diabetes has been held by different writers to affect each of the things which it is known can be done with sugar by a normal body, *e.g.*, to burn it, to produce it in moderation, to store it as fat, or to "fix" it as glycogen, etc. As far back as 1871 Schultzen suggested that the primary error was an inability to open up the glucose molecule and so render it fit for oxidation.

For a number of years we have found it serviceable to regard dissociation of sugar as prerequisite for its chemical reactivity in general, *i.e.*, for its oxidation, reduction, polymerization, combination, or functioning in equilibria—in the same sense that this has been made the basis for a great array of organic chemical reactions *in vitro* by J. U. Nef. The active principle in the so-called internal secretion of the pancreas is a substance whose effect, like that of alkali *in vitro*, is to increase the dissociation of certain classes of substances including the sugars. Once dissociated, the fate of the residues thus set free depends upon the reaction conditions encountered by these residues in the various cells and fluids of the body.

Sarcolactic acid has a peculiar interest in this connection because it may be formed in the body under certain circumstances (*i.e.*, under conditions tending toward asphyxia—general or local), and because in so far as it comes from sugar it represents purely and simply a product of dissociation and intramolecular rearrangement. This interpretation of lactic acid as a dissociation product of glucose has been made by a number of writers. The question arises: Is the power of the body to form lactic acid out of sugar lessened in diabetes?

It is a fact that lactic acid is seldom found in detectable quantities in the ether extracts so frequently obtained in the routine determination of β -hydroxybutyric acid in the urine of severe diabetes. Von Noorden¹ states that he has three times sought it in vain. We have also failed to prepare an insoluble zinc salt from this residue in a number of attempts. The reason for this is clear when one recalls the work of Mandel and Lusk² who demonstrated the complete conversion of administered lactic acid into sugar in fully phlorhizinized dogs and its failure to occur even in the urine of dogs which were poisoned with phosphorus when these dogs were fully phlorhizinized. Obviously one can draw no conclusion from the urinary findings as to the diabetic's ability to form lactic acid. There are other ways, however, of attacking the problem.

Fletcher and Hopkins³ have held that the lactic acid which is formed in fresh muscles, and which has been regarded by many as intimately connected with rigor mortis and death, is a product solely of the survival period. Under proper circumstances a given weight of muscle will produce a certain maximum quantity of lactic acid, after which no injury or stimulation will influence it to form any more. (Lactic acid can also be formed according to Kondo⁴ by the action of fresh muscle juice and the quantity formed can be varied by altering the reaction of the juice. The addition of alkali according to him prolongs and increases, the addition of acid shortens and decreases the lactic acid formation. He believes that the degree of acidity may set the limit for survival lactic acid formation. But this does not imply that with a given surviving muscle the quantity of lactic acid produced is not a function of the mother substance from which it arises.) Now since the lactic acid formed in an isolated muscle is not subjected to the drain of sugar which occurs during life when glycosuria is in progress and which tends to "draw" into glucose the lactic acid, alanine, glycerol, and other substances which are normally in equilibrium with glucose, we decided to produce diabetes in animals, study their metabolism during life, and then ascertain the power of the isolated muscle to form lactic acid during the survival period.

¹ *Handbuch der Pathologie des Stoffwechsels*, 1907, ii, p. 95.

² Mandel and Lusk: *Amer. Journ. of Physiol.*, xvi, p. 129, 1906.

³ *Journ. of Physiol.*, xxxv, p. 247, 1907.

⁴ *Biochem. Zeitschr.*, xlv, p. 63, 1912.

Methods.

The method employed for securing a maximum survival yield of lactic acid was devised in accordance with the principles established in the papers of Fletcher and Hopkins⁵ and of Fletcher.⁶ According to them mammalian muscles are tardy in reaching their final acidity unless hurried by methods which provide a suitable temperature and stimulus without premature injury. Absence of oxygen, the proper time and temperature, certain chemicals, *e.g.*, chloroform, insure completeness of the process. The animals used in these experiments were deeply anaesthetized with chloroform. The abdomen was then opened and portions of liver rapidly removed for glycogen determinations, which were carried out in accordance with the directions of Pflüger.⁷ The great abdominal vessels were then slashed and the animal suspended by the hind limbs to insure as complete exsanguination of the latter parts as possible. Muscle was then rapidly dissected from the fore-quarters for glycogen determination. This done, the carcass was immersed in water at 45°C. for an hour, then removed from the water and kept in a covered boiler for six hours more at 30–35°C. Rigor mortis always developed fully during the first hour. At the end of the six hours the skin was dissected from the hind limbs, after which the muscles were dissected out cleanly, care being taken to use the same groups on each side. The muscles were then run through a mincer, after which the minced muscle was thoroughly mixed. The mincing and mixing were done four times in all, after which the hash was weighed out into 50-gram portions, each of which was covered with 500 cc. of 95 per cent alcohol.

The subsequent steps in the alcohol extraction, the removal of fat and lipoids by means of blood charcoal, the ether extraction out of the acidified aqueous solution, the conversion of the lactic acid into the zinc salt, its weighing in the anhydrous form, as well as the determination of its purity by means of ZnO determinations, were all done essentially as described by Fletcher and Hopkins. We introduced, however, certain mechanical changes.

Thus, in the original alcohol extraction we simply stirred the hashed muscle with a rod until all clumps were thoroughly separated and after

⁵ *Journ. of Physiol.*, xxxv, p. 247, 1907.

⁶ *Ibid.*, xliii, p. 286, 1911.

⁷ Abderhalden: *Handbuch der Biochem. Arbeitsmethoden*, 1910, ii, p. 1071.

twenty-four hours' standing decanted the alcohol on to a small, folded filter. (Fletcher and Hopkins rubbed up the muscle and alcohol in a mortar and squeezed out the muscle mass in muslin after decanting, making, in all, four extractions.) After five extractions, each with 500 cc. of 95 per cent alcohol, the muscle residue was allowed to dry in a desiccator, then pulverized in a mortar and finally extracted twice more as before. In clearing with blood charcoal to remove lipoids, etc., we made the aqueous solution of the alcoholic residue to exactly 150 cc., then added 7-10 grams of blood charcoal and carried out the heating under an efficient reflux condenser. After cooling, the liquid was filtered, and an aliquot part of the whole was used for analysis, generally 120 cc. or 80 per cent. This avoided the process of washing and reboiling the charcoal, employed by Fletcher. The filtrate was then evaporated to dryness, made up with H_2O to exactly 20 cc. in a bulbed graduate ("phenol tube") and then to 25 cc. with a saturated solution of phosphoric acid. This solution was covered with 100 cc. of ether and shaken. In separating the ether layer we employed a special siphon instead of a pipette and carried out the extraction as stated in a "phenol tube," the bulb of which permitted the use of a large volume of ether for the 25 cc. of solution, although the graduated stem of the tube was narrow and permitted of a very sharp separation of layers. We extracted the aqueous residue ten times with exactly 100 cc. of ether each time, and proceeded in the same way in each experiment. The ether subsequent to decantation was handled as in Magnus-Levy's method for β -hydroxybutyric acid determination in the urine, to ensure freedom from emulsified solution containing inorganic acid. The final zinc salt was always white and pure and when analyzed gave uniformly satisfactory ZnO figures, so that in some experiments we omitted this final ignition. Analyses were made in pairs.

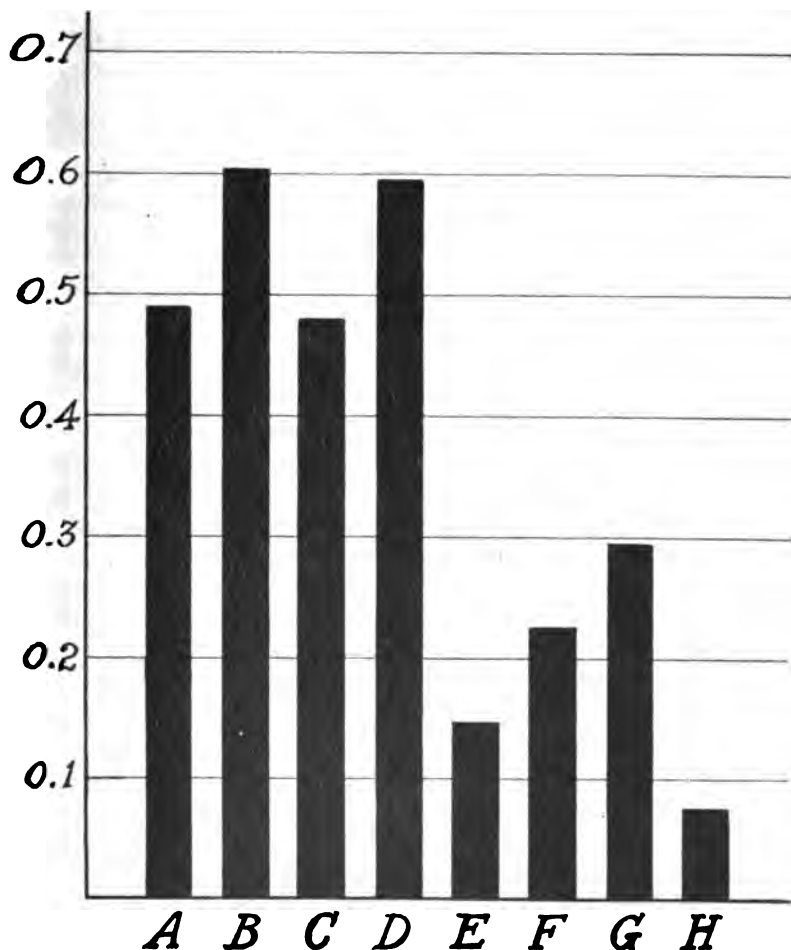
Phlorhizinization of dogs was accomplished by administering to animals of 7-9 kgms. body weight, every 6 hours subcutaneously, 2 grams of phlorhizin dissolved in 30 cc. of 1.2 per cent solution of sodium carbonate. During the first hours of the regimen the dogs were given cold baths and permitted to shiver afterwards, according to Lusk's method. The urine was obtained by catheter and the bladder irrigated with warm water at the end of each 6-hour period. Nitrogen determinations in the urine were made by the Kjeldahl method, dextrose by polariscope, and by the titration method of Bang and Bohmannsson.⁵

Results.

The relative quantities of lactic acid obtained per 100 grams muscle, and expressed in weights of anhydrous zinc lactate may be seen by reference to the chart. The first four lines, A, B, C,

⁵ *Zeitschr. f. physiol. Chem.*, lxiii, p. 443, 1909.

and *D*, represent the quantity of zinc lactate obtained from the lactic acid in 100 grams of muscle from healthy dogs, in such states of nutrition as they were when brought to the laboratory. The last line *H* is the figure obtained in a case of severe human diabetes. The other three lines, *E*, *F*, *G*, correspond to the figures obtained from muscles of phlorhizinized dogs, the lowest, *E*, being that from a fully phlorhizinized and glycogen-free animal in which the D:N ratio was 3.73 : 1; the next lowest, *F*, a dog in which the ratio was 2.82:1 and in which a trace of glycogen was found; the highest



of the three, *G*, a dog in which the ratio was 2.90 : 1, but to which glucose was given intravenously just before death in order to create an hyperglycaemia, and which contained in the muscles 0.22 per cent glycogen.

Discussion of results.

It is apparent from these figures that the quantity of lactic acid formed in muscles from healthy dogs varies, presumably with the different states of nutrition. It is also clear that the muscle from the human diabetic formed distinctly less lactic acid than the lowest normal muscle. But it is very possible that the survival lactic acid comes from three sources: (1) glycogen sugar, (2) sugar derived from protein or fat, (3) directly from fatty or amino-acids without intermediate sugar formation. In the case of the muscle of the human diabetic, the fraction (1) from glycogen might naturally be expected to be absent or very low because in such diabetics the muscles contain little or no glycogen. Therefore the muscle of the human diabetic had presumably less material to work upon than the normal and the values obtained are not directly comparable to those found for muscles of well nourished animals. In Experiment E the factor (1) was removed by means of phlorhizin, thus producing a state of nutrition parallel to that found in human diabetes but nevertheless without injury to the pancreas. Incidentally an hypoglycaemia was caused, *i.e.*, the value of (2) was somewhat lowered. The lactic acid formed by the muscle of this dog is therefore directly comparable to that found in the human, except for the difference in species and perhaps in the lessened value of (2).

In Experiment F the attempt was made to replace (2) by creating hyperglycaemia just before death, but in such experiments there is a rise both in the amount of glycogen and of lactic acid found. This experiment appears to demonstrate directly the power of the muscle to form lactic acid and glycogen from glucose, and affords possibly the best comparison of the series with the muscle of the human diabetic. Now if we compare the lactic acid formed in human diabetes with that formed in the phlorhizinized dogs (in which pancreatic function was normal) it appears that with the same material at hand out of which to form lactic acid or even with a little less at hand, the phlorhizinized

dog muscle made somewhat more lactic acid than that of the **human** diabetic.

In all cases in which diabetic muscles were analyzed there was some lactic acid formation. This would have to be ascribed to **protein** (and perhaps in part to fat)—either directly or through **sugar** as an intermediate. Owing to this fact—that lactic acid formed in surviving muscles comes partly from sources other than **sugar**—lactic acid cannot be made a direct measure of the disassociation of sugar in these experiments as was originally hoped.

As an incident it is interesting to note that a dog, in which a **D:N** ratio of 3.73:1 was produced, proved to be in reality glycogen-free by post-mortem analysis, a confirmation of the conclusion reached by Lusk.⁹ The animal in which the ratio was 2.83 showed a trace of glycogen in the muscles. And with a ratio of 2.97 and the intravenous administration of glucose the glycogen in the muscle was 0.22 per cent.

EXPERIMENTS.

I.

Experiments with healthy dogs in different states of nutrition handled in accordance with methods described on p. 445.

EX- PERI- MENT	TOTAL $\text{Zn}(\text{C}_4\text{H}_5\text{O}_2)_2$ (ANHYDROUS) PER 100 GMS. MUSCLE	AMOUNT $\text{Zn}(\text{C}_4\text{H}_5\text{O}_2)_2$ USED FOR INCINERATION	ZnO		
			Weight obtained	Per cent	Theory
A	0.491	0.321	0.109	34.24	33.43
B	0.605				
C	0.481				
D	0.597	0.235	0.085	36.17	33.43

⁹ *Elements of the Science of Nutrition*, 1909, second edition.

II.

Experiments with phlorhizinized dogs—phlorhizinized and handled as described under Methods, p. 444.

EXPERIMENT E. The urinary findings for successive 6-hour periods gave the following figures. (Dextrose, Bang-Bohmannsson; N, Kjeldahl.)

PERIOD	DEXTROSE	N	D:N	AVERAGE D:N FOR LAST 18 HOURS
	<i>per cent</i>	<i>per cent</i>		
I	2.50	0.35	7.14	} 3.73 : 1
II	4.04	0.57	7.09	
III	2.90	0.58	5.00	
IV	3.50	0.97	3.60	
V	3.86	0.96	4.00	
VI	3.59	1.00	3.59	

Glycogen determinations: carried out on 50 grams each of muscle and liver (Pflüger). The final residue obtained after filtration of the alcohol was dissolved in 20 cc. of hot water and cleared with HCl as per Pflüger's directions. This fluid showed no dextro-rotation and, after boiling for one-half hour with 10 per cent HCl and neutralization, no reducing power in Haines' solution. The iodine reaction was also negative.

Total anhydrous $Zn(C_2H_3O_2)_2$ obtained per 100 grams muscle, 0.1467 gram. Used for ZnO determination, 0.0514 gram. ZnO found, 0.0172—corresponding to 33.46 per cent; theory, 33.43 per cent. A blood-sugar determination (Bang, Lyttkens and Sandgren, *Zeitschr. f. physiol. Chem.*, lxx, p. 497) showed 0.073 per cent.

EXPERIMENT F. *Phlorhizinized dog.* The same procedure as in E. except that no effort was made to collect the whole urine for each period nor to maintain even dilution of the urine.

PERIOD	DEXTROSE	N	D:N	AVERAGE D:N FOR LAST 18 HOURS
	<i>per cent</i>	<i>per cent</i>		
I				} 2.82 : 1
II	2.55	0.46	5.59	
III	1.93	0.67	2.88	
IV	1.82	0.69	2.64	
V	1.80	0.61	2.94	

Glycogen determination: in muscle, trace; in liver, trace (?).

Total anhydrous $Zn(C_2H_3O_2)_2$ per 100 grams muscle, 0.227 gram. Used for ZnO determination, 0.0782 gram. ZnO found, 0.0263, corresponding to 33.65 per cent; theory, 33.43 per cent.

EXPERIMENT G. *Phlorhizinized dog with intravenous glucose injection.*

PERIOD	DEXTROSE	N	D:N	AVERAGE D:N FOR LAST 18 HOURS
	<i>per cent</i>	<i>per cent</i>		
II	4.17	0.85	4.85	2.97
III	4.17	1.57	2.66	
IV	2.50	0.74	3.37	
V	2.40	0.83	2.90	

At the close of the last period analyses of the urine were made as usual, the time consumed being two hours. The animal was then chloroformed and 100 cc. of a 12.9 per cent glucose solution introduced into the femoral vein. The ureters were then clamped to stop excretion. Ten minutes later the usual procedure was carried out.

Glycogen determination: in liver, a trace (the residue from 50 grams liver, made up in the final step to 50 cc. with water, read in a 20 cm. tube + 0.06°). Glycogen in the muscle, 0.219 per cent (final residue from 50 grams of muscle made up to 20 cc. read in 20 cm. tube + 1.09°).

III.

Human diabetes (so-called "bronze diabetes").

EXPERIMENT H. *Clinical history:* Dr. G. P., age 42; case No. 61501, Presbyterian Hospital, Chicago. Service of Dr. Frank Billings. Patient entered the hospital June 15, 1911, and died in true dyspnoeic coma ten weeks later. He probably had a transient glycosuria four years prior to admission. Four months before admission a controllable glycosuria occurred. One month before admission it became intractable. On admission the urine contained much sugar, acetone, acetoacetic and β -hydroxybutyric acids with a high NH_4 figure. He complained of weakness, loss of weight and strength, thirst and polyuria. Just before this time because of an old luetic affair he had taken a course of baths and mercurial inunctions after which he was troubled greatly with pain in the distribution of the sacral nerves.

Physical examination on admission. Patient is 5 feet 10 inches tall and weighs only 102 pounds. The skin is a little dusky—suggesting pigmentation. The liver presents a hard, sharp and smooth edge 2.5 inches below the costal arch in the mamillary line. The tip of the spleen is palpable. The arteries are somewhat sclerosed. Otherwise the findings have no bearing in this place. Wassermann, negative.

Subsequent history. He was frequently in trouble from the start with headache, slight nausea, drowsiness, backache and a rising acidosis if he attempted to move far from his bed or room.

At one time he was given an analyzed diet—containing protein, 85 grams; wheat starch, 75 grams (or 100 grams as noted); fat, 170 grams; aggregating

(approximately) 2170 Cal. In addition to this he received sodium bicarbonate, 20 grams, and claret, 400 cc. *per diem*.

The findings follow:

DAY	DIET	WEIGHT pounds	AMOUNT	SPECIFIC GRAVITY	DEXTROSE					
					Per cent	Total	NH ₄	N	Q	D, N
23	As stated above.....	105	4330	1025	2.43	105	4.4	13.4	852	2
24	Same.....	105	4425	1027	2.53	111	3.5	18.6	771	9
25	Same except starch made to 100 gm....	105	4725	1025	2.82	133	3.6	19.0	781	7
27	Same as on 25.....	105	4700	1025	2.93	133	7.5	18.9	771	7
28	Interrupted.....	105	5100	1026			8.1			

$$Q = \frac{\text{Urinary glucose} \times 100}{(\text{Urinary N} \times 3.65) + \text{carbohydrate ingested}}$$

$$\frac{D}{N} = \frac{\text{Urinary glucose} - \text{carbohydrate ingested}}{\text{Urinary N}}$$

On the 26th he ran a slight temperature ascribed to exacerbation of an old prostatitis and on the 27th the NH₄ figure was extreme. A fast day was therefore interpolated with a simultaneous increase of the wine and alkali and addition of opium. There was temporary improvement but thereafter his condition was always critical and demanded frequent alterations of the diet. Analysis of the food, etc., was consequently discontinued. The usual clinical resources were employed. He could be held in N equilibrium but not without the ingestion of much fat and the appearance of threatening symptoms. From August 12 to 20, his diet averaged approximately 100 grams of carbohydrate, 125 grams protein, 200 grams fat, supplemented with wine, 0.5 to 1 liter, and sodium bicarbonate, 15 to 20 grams. The routine examination of the urine for this period ran as follows:

DAY	AMOUNT URINE	SPECIFIC GRAVITY	GLUCOSE	NH ₄
12	3950	1019	71	2.8
13	3000	1026	108	1.9
14	3800	1026	150	1.9
15	4325	1029	164	2.4
16	3500	1026	108	2.3
17	3800	1030	144	2.5
18	3425	1030	145	2.5
19	4300	1032	168	3.0
20	4450	1030	165	3.9

Assuming the daily average carbohydrate ingestion to have been 100 grams and the average urinary N to have been 20 grams (which cannot be far from what occurred) the Q for the last nine days would have been 77 as in the previous period. *The case represents therefore a "severe" but "incomplete" diabetes.*

Autopsy. Performed four hours after death. Body still warm. The liver and pancreas show the typical sclerosis and brick-red parenchyma of "bronze diabetes." Psoas muscle taken for analysis was kept four hours in a warm place, then handled as usual.

Total anhydrous Zn(C₂H₃O₂)₂ per 100 grams muscle, 0.077 gram.

SUMMARY.

Muscles of glycogen-free animals form some sarcolactic acid (about 30 per cent of the normal). This lactic acid cannot come from glycogen but must arise from preformed sugar, or directly from certain amino- or fatty-acids. The muscle of a case of severe human diabetes formed even less lactic acid than that of fully phlorhizinized dogs. This suggests an impaired power to dissociate glucose on the part of the diabetic muscle since such muscles are bathed with an abnormally high quantity of sugar which—if available—should yield more lactic acid than is found in the muscle of phlorhizin diabetes.

With D:N ratios of approximately 3.65 : 1, post-mortem analyses of dog muscles and livers show no glycogen. With ratios of 2.8 or 3.0 : 1 this is not necessarily the case, and one cannot assume that with a constant D : N ratio of 2.8 : 1 an animal is free of glycogen.



ON THE ABSORPTION OF NITROGENOUS PRODUCTS— A REPLY TO ABDERHALDEN AND LAMPÉ.

BY OTTO FOLIN AND W. DENIS.

(*From the Biochemical Laboratory of Harvard Medical School, Boston.*)

(Received for publication, April 9, 1913.)

Some time ago Abderhalden and Lampé published a paper¹ on the absorption of amino-acids, peptones, etc., which is largely devoted to a review and criticism of our experimental results and conclusions on the same subject.²

In this paper Abderhalden presents his views as to the nature of the problem, the relative merits of the theories in vogue with regard to absorption, the difficulties involved, etc., and reviews the bearing on these questions of his own researches including some new experiments which so far as can be judged from the rather sketchy description and the protocols, represent qualitative control experiments of our absorption determinations. The results recorded we interpret as a very acceptable confirmation of our facts and interpretations. So far as our work is concerned, however, Abderhalden admits that our analytical methods are new, but considers the results which we obtained neither new nor particularly illuminating.

The theory of amino-acid absorption accompanied by immediate deamination is according to Abderhalden superfluous to discuss because it lacks experimental support. (Our experiments have shown the immediate deamination hypothesis to be untenable.) The theory of amino-acid absorption and subsequent distribution to the different tissues as presented in our papers Abderhalden considers not yet proven; in other words, this is still a theory and not an established fact. Even as a theory he is inclined to be more critical of it than of his own "Arbeitshypothese"—the immediate regeneration of blood proteins out of the amino-acids. The

¹ *Zeitschr. f. physiol. Chem.*, lxxxi, p. 473, 1912.

² This *Journal*, xi, p. 87, 1912; xii, p. 141, 1912.

regeneration doctrine, though it too is only a hypothesis and without a particle of positive experimental evidence in its favor, he nevertheless thinks should be supported until *facts* have made it superfluous (pp. 495-6). Just why this one hypothesis should receive the benefit of all remaining doubts he does not make clear, but since he still takes that position others, like ourselves, feel justified in referring to him as the chief exponent and defender of it. The fact that he in "1912" mentioned its weakness from an experimental standpoint (Folin made clear its weakness in 1905)² does not alter the fact that it is still his working hypothesis, though perhaps in a modified form. If we understand him rightly his working hypothesis now is that those amino-acids which cannot be built up into blood protein may get into the blood as amino-acids.

While Abderhalden thus remains avowedly a supporter of the immediate regeneration hypothesis as a feature of absorption it is clear that his experimental results described in his paper with Lampé confirm our findings so far as such qualitative tests can confirm them. By qualitative tests using 10 cc. of blood he is now able to demonstrate the presence of amino-acids in the blood at all times and an unmistakable increase in the reaction as a result of a few minutes' absorption of any ordinary amino-acid. In his earlier work Abderhalden was unable to prove the presence of amino-acids in blood even when he worked with as much as 50 liters at a time.

Abderhalden's severely critical attitude toward our results and interpretations appears to be a consequence of his bias in another direction. To those who for years have recognized that the protein regeneration doctrine had only an old teleological reason, based on false premises, to support it, and who consequently regarded the immediate deaminization theory as more probable and reasonable, our facts and interpretations will appear in a different light.

As for ourselves, we hold that our experimental results transform into a demonstrable fact the theory of amino-acid absorption unaccompanied by immediate deaminization or protein regeneration. We recognize that our facts need verification, and any one who will learn to use our quantitative methods can easily repeat our work.

² *Amer. Journ. of Physiol.*, xiii, p. 117, 1905.

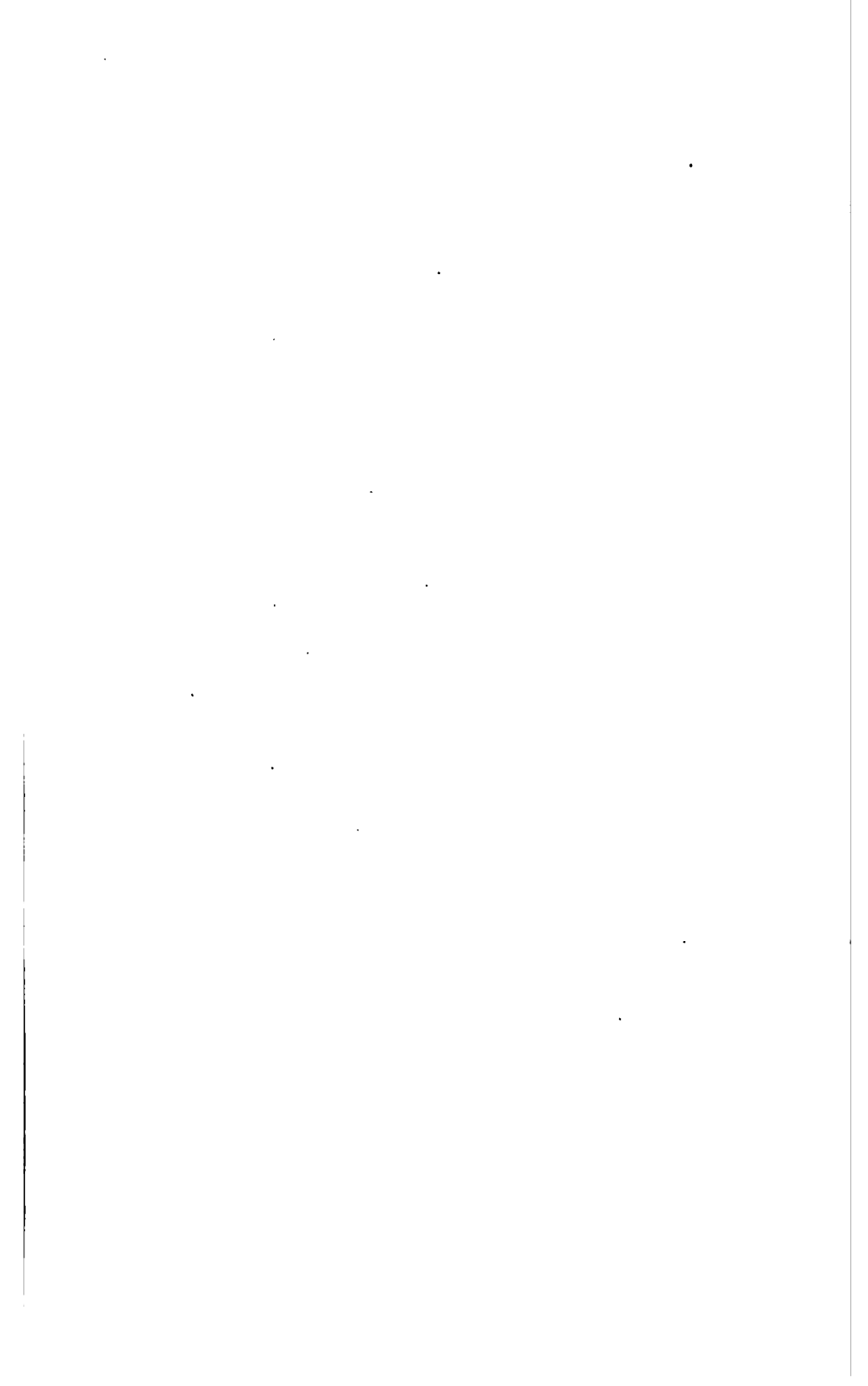
Abderhalden constantly overlooks the *quantitative* aspect of our results as when he says that these have no more significance than has the presence of traces of glycerin in the blood in connection with the problem of fat absorption, or when he says that we must apply his and Lampé's qualitative "indirekten Bestimmungen" (p. 491) in order to obtain a correct interpretation of our results.

By the help of Abderhalden's favorite reagent, triketo hydrin hydrate, or nin hydrin as he now calls it, we have no doubt that additional interesting confirmatory tests may be obtained, and we hope that he will continue to use it, and that he will also apply it to the study of the absorption of amino-acids from the stomach.⁴ Since there is not any reason direct or indirect, theoretical or experimental, for assuming that our quantitative results on the absorption and distribution of amino-acids are less valid than our corresponding results with urea, creatine and creatinine, we regard the qualitative tests demanded by Abderhalden as interesting and valuable to be sure, but nevertheless only as merely confirmatory. In the case of one amino-acid, tyrosine, we did substitute a qualitative test and by its help were able to trace its passage into the blood and muscles, a fact which Abderhalden seems to have overlooked.

In conclusion we think that Abderhalden interpreted somewhat unfairly the reservation of the field opened by our technique. The reservation manifestly referred only to our methods of studying the problem, *i.e.*, by the help of our new analytical methods. And it was evidently thus understood by Van Slyke and Meyer,⁵ the first to bring out facts which verify our findings. Moreover in the paper describing the method, only three months later, a reprint of which was in Abderhalden's possession, we revoked the reservation in the most unmistakable manner: "We hereby expressly revoke our earlier reservation of the field of research referred to in those papers by means of these methods."

⁴ Comment on Abderhalden's criticisms on the demonstration of absorption from the stomach by Folin and Lyman is superfluous since in a reply to London (this *Journal*, xiii, p. 389, 1912) that matter was adequately covered.

⁵ This *Journal*, xii, p. 399, 1912.



ON THE TYROSINE CONTENT OF PROTEINS— A REPLY TO ABDERHALDEN AND FUCHS.

BY OTTO FOLIN AND W. DENIS.

(*From the Biochemical Laboratory of Harvard Medical School, Boston.*)

(Received for publication, April 9, 1913.)

In a recent paper by Abderhalden and Fuchs,¹ entitled "Über den Gehalt der Proteine an l-Tyrosin und die Genauigkeit der Bestimmung dieser Aminosäure" the closing sentence reads as follows:

"Die kolorimetrische Methode von Folin und Denis vermag die Bestimmung des Tyrosins durch Krystallisation nicht zu ersetzen, weil sie auch andere Aminosäuren nachweist und infolgedessen zu hohe Werte liefert."

The statement contained in the above sentence is given as though it embodied a conclusion derived from the experimental work described in the paper whereas in point of fact it represents only an opinion. The only observation recorded by Abderhalden and Fuchs in support of that opinion is that tryptophane and a certain obscure substance ("oxytryptophan") which, so far as we know no one but Abderhalden has yet observed, also give a slowly developing blue color with our tyrosine reagent.

We omitted to apply our color reaction to tryptophane partly because we had none on hand and partly because we assumed (perhaps incorrectly) that very little tryptophane would survive the treatment with boiling mineral acids which we employed for splitting off the tyrosine. Further, it is to be noted that the excess of tyrosine indicated by our method is no greater in the case of casein with 1.5 per cent tryptophane than in the case of zein which is supposed to have none. For the former we obtained 6.5 per cent as against 4.5 per cent obtained by E. Fischer, for the latter our figure is 5.5 per cent as against Osborne's 3.6 per cent.

¹ *Zeitschr. f. physiol. Chem.*, lxxviii, p. 468, 1913.

On the strength of Abderhalden's observation we nevertheless concede for the time being at least that our tyrosine figures may be somewhat high though there is clearly no reason to believe that an adequate explanation of the difference between our figures and those recorded in the literature are to be explained by the presence of tryptophane. Our comparisons were made with the tyrosine figures already in the literature including several of Abderhalden's. If Abderhalden and Fuchs have now (for the first time) succeeded in working out a method for a quantitative isolation of pure tyrosine, that is a different matter, and when Abderhalden on the basis of his new method has published revised figures for the tyrosine content of at least some proteins, we shall perhaps know whether our figures represent tyrosine or tyrosine plus something else. Until he has done so, however, we would rather insist that our tyrosine figures are more nearly correct than those heretofore recorded in the literature.

THE INFLUENCE OF BASES UPON THE RATE OF OXIDATIONS IN FERTILIZED EGGS.

BY JACQUES LOEB AND HARDOLPH WASTENEYS.

(From the Rockefeller Institute for Medical Research, New York.)

(Received for publication, April 18, 1913.)

1. In a previous paper we have shown that the weak bases cause a more rapid and greater increase in the rate of oxidations in the *unfertilized egg* than the strong bases.¹ This result differed in principle from the results which Warburg obtained while working on *fertilized eggs*.² He found that NH_4OH does not increase the rate of oxidations in *fertilized eggs* of *Strongylocentrotus lividus* at Naples, while NaOH does; and since NH_4OH diffuses into the egg while NaOH does not, he concludes from this that the effect of OH ions on oxidations is neither determined by their diffusion into the egg nor through their reaction with the plasma membrane, but merely through their presence in the solution surrounding the cell (p. 316). This conclusion does not harmonize with the results which we obtained with the unfertilized egg, in which NH_4OH influenced the oxidations more strongly than the NaOH . We therefore made some experiments on *fertilized eggs* in order to clear up if possible this apparent contradiction. This seemed sufficiently important since it affects not only the theory of fertilization but also the problem of the localization of oxidations in the cell.

Warburg only published the results of three determinations of the rate of oxidations at three different concentrations of NaOH in a mixture of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ in that proportion in which these salts occur in the sea water. His results were as follows:

TABLE I.

Rate of oxidations in fertilized eggs of *Strongylocentrotus lividus* at Naples.

C_{NaOH}	COEFFICIENT OF OXIDATIONS	
10^{-8}N	1.4	No segmentation.
10^{-6}N	3.9	Normal segmentation.
10^{-4}N	8.1	No segmentation.

¹ Loeb and Wasteneys: this *Journal*, xiv, p. 355, 1913.

² O. Warburg: *Zeitschr. f. physiol. Chem.*, lxvi, p. 305, 1910.

It seemed of importance to us to ascertain whether indeed the rate of oxidations in fertilized eggs increased steadily with the increase in the concentration of NaOH. For this reason experiments were carried on with the fertilized eggs of *Strongylocentrotus purpuratus* in California. Of freshly fertilized eggs of one female a homogeneous suspension was made and this was divided into five equal lots which were then distributed into five different solutions of NaCl + KCl + CaCl₂ (in the proportion in which these salts exist in the sea water) and various quantities of $\frac{N}{10}$ NaOH were added.

TABLE II.

NATURE OF THE SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm.	
Neutral NaCl + KCl + CaCl ₂	0.44	1.00
50 cc. NaCl + KCl + CaCl ₂ + 0.3 cc. $\frac{N}{10}$ NaOH	0.48	1.08
50 cc. NaCl + KCl + CaCl ₂ + 0.4 cc. $\frac{N}{10}$ NaOH	0.48	1.06
50 cc. NaCl + KCl + CaCl ₂ + 0.5 cc. $\frac{N}{10}$ NaOH	0.50	1.13
50 cc. NaCl + KCl + CaCl ₂ + 0.6 cc. $\frac{N}{10}$ NaOH	0.52	1.18
Neutral NaCl + KCl + CaCl ₂	0.47	1.00
50 cc. NaCl + KCl + CaCl ₂ + 0.7 cc. $\frac{N}{10}$ NaOH	0.55	1.17
50 cc. NaCl + KCl + CaCl ₂ + 0.8 cc. $\frac{N}{10}$ NaOH	0.71(?)	1.51(?)
50 cc. NaCl + KCl + CaCl ₂ + 0.9 cc. $\frac{N}{10}$ NaOH	0.68	1.44
50 cc. NaCl + KCl + CaCl ₂ + 1.0 cc. $\frac{N}{10}$ NaOH	0.73	1.55
Neutral NaCl + KCl + CaCl ₂ (two determina- tions).....	0.41 0.43	
50 cc. NaCl + KCl + CaCl ₂ + 1.1 cc. $\frac{N}{10}$ NaOH	0.76	1.81
50 cc. NaCl + KCl + CaCl ₂ + 1.2 cc. $\frac{N}{10}$ NaOH	0.81	1.93
50 cc. NaCl + KCl + CaCl ₂ + 1.3 cc. $\frac{N}{10}$ NaOH	0.89	2.12
50 cc. NaCl + KCl + CaCl ₂ + 1.4 cc. $\frac{N}{10}$ NaOH	0.79	(eggs injured)

These results seem to indicate one point clearly, namely, that the addition of 0.5 cc. of $\frac{N}{10}$ NaOH or less to 50 cc. of salt solution has practically no effect upon the rate of oxidations. This experiment was repeated (table III).

The experiment confirms the result of the preceding series. The addition of 0.4 cc. or less of $\frac{N}{10}$ NaOH to 50 cc. of salt solution has

no effect upon the rate of oxidations in the fertilized egg, and the addition of 0.8 cc. of $\frac{N}{10}$ NaOH has only a very slight effect.

TABLE III.

NATURE OF THE SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm.	
Neutral NaCl + KCl + CaCl ₂	0.58	1.00
50 cc. NaCl + KCl + CaCl ₂ + 0.1 cc. $\frac{N}{10}$ NaOH	0.58	1.00
50 cc. NaCl + KCl + CaCl ₂ + 0.2 cc. $\frac{N}{10}$ NaOH	0.53	0.90
50 cc. NaCl + KCl + CaCl ₂ + 0.4 cc. $\frac{N}{10}$ NaOH	0.60	1.00
50 cc. NaCl + KCl + CaCl ₂ + 0.8 cc. $\frac{N}{10}$ NaOH	0.83	1.40

We next tried the effect of another strong base, namely, tetraethylammoniumhydroxide, on the rate of oxidations in fertilized eggs of *S. purpuratus*. The following table gives the result.

TABLE IV.

NATURE OF THE SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm.	
Neutral NaCl + KCl + CaCl ₂	0.45	1.00
50 cc. NaCl + KCl + CaCl ₂ + 0.2 cc. $\frac{N}{10}$ N(C ₂ H ₅) ₄ OH	0.44	1.00
50 cc. NaCl + KCl + CaCl ₂ + 0.4 cc. $\frac{N}{10}$ N(C ₂ H ₅) ₄ OH	0.44	1.00
50 cc. NaCl + KCl + CaCl ₂ + 0.5 cc. $\frac{N}{10}$ N(C ₂ H ₅) ₄ OH	0.47	1.00

The addition of 0.5 cc. of $\frac{N}{10}$ N(C₂H₅)₄OH to 50 cc. of salt solution does not influence the rate of oxidation in the fertilized eggs of *S. purpuratus*. The addition of more N(C₂H₅)₄OH would interfere too much with the analytical result and was not undertaken.

2. Weak bases influence the rate of oxidations in the fertilized egg of *Strongylocentrotus purpuratus* but slightly. This is shown in table V for NH₄OH and in table VI for methylamine.

This result agrees with the observation of Warburg.

3. We have to explain two groups of facts:

1. The apparent difference in the action of weak and strong bases on fertilized eggs.

2. The difference in the reaction of fertilized and unfertilized eggs. We will take up these questions in succession. The fact

Oxidations in Fertilized Eggs

TABLE V.

NATURE OF THE SOLUTION	OXYGEN CONSUMED	PERCENTAGE OF OXIDATION
Control NaCl + KCl + CaCl ₂ { a.	0.43	
b.	0.47	
e. NaCl + KCl + CaCl ₂ + 0.6 cc. $\frac{N}{10}$ NH ₄ OH.	0.66	1.47
e. NaCl + KCl + CaCl ₂ + 1.0 cc. $\frac{N}{10}$ NH ₄ OH.	0.65	1.41
e. NaCl + KCl + CaCl ₂ + 1.4 cc. $\frac{N}{10}$ NH ₄ OH.	0.61	1.36
e. NaCl + KCl + CaCl ₂ + 1.6 cc. $\frac{N}{10}$ NH ₄ OH.	0.59	1.31
e. NaCl + KCl + CaCl ₂ + 1.8 cc. $\frac{N}{10}$ NH ₄ OH.	0.63	1.40
e. NaCl + KCl + CaCl ₂ + 2.0 cc. $\frac{N}{10}$ NH ₄ OH.	0.61	1.36
Control NaCl + KCl + CaCl ₂	0.55	
e. NaCl + KCl + CaCl ₂ + 0.1 cc. $\frac{N}{10}$ NH ₄ OH.	0.60	1.09
e. NaCl + KCl + CaCl ₂ + 0.3 cc. $\frac{N}{10}$ NH ₄ OH.	0.74	1.35
e. NaCl + KCl + CaCl ₂ + 0.6 cc. $\frac{N}{10}$ NH ₄ OH.	0.69	1.25
e. NaCl + KCl + CaCl ₂ + 1.0 cc. $\frac{N}{10}$ NH ₄ OH.	0.74	1.35
e. NaCl + KCl + CaCl ₂ + 1.8 cc. $\frac{N}{10}$ NH ₄ OH.	0.70	1.27
e. NaCl + KCl + CaCl ₂ + 2.4 cc. $\frac{N}{10}$ NH ₄ OH.	0.72	1.31
e. NaCl + KCl + CaCl ₂ + 3.0 cc. $\frac{N}{10}$ NH ₄ OH.	0.73	1.32

TABLE VI.

NATURE OF THE SOLUTION	OXYGEN CONSUMED
Control NaCl + KCl + CaCl ₂	0.41
e. NaCl + KCl + CaCl ₂ + 0.4 cc. $\frac{N}{10}$ methyl- e.	0.46
e. NaCl + KCl + CaCl ₂ + 0.6 cc. $\frac{N}{10}$ methyl- e.	0.44
e. NaCl + KCl + CaCl ₂ + 0.8 cc. $\frac{N}{10}$ methyl- e.	0.40
e. NaCl + KCl + CaCl ₂	0.42

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that weak bases have no effect upon the fertilized eggs of *purpuratus* finds its explanation in the fact that strong bases do not act either except in concentrations above $10^{-5}N$. If we assume that this effect is due to the action of OH ions it would require a concentration of OH ions more to produce a similar effect, since the concentration of NH₄OH is so much lower than that of NaOH. It seems very important to point out that the conclusion from this experiment upon the effect of bases in the egg, since that concentration above $10^{-5}N$ raises the rate of oxidations in the fertilized egg, is that the maximum concentration of NaOH to the egg to develop normally (in a mixture of 10 cc. of NaOH and 90 cc. of solution). The rate of oxidation is found that it varies somewhat between 0.1 cc. of NaOH to 50 cc. of solution. The rate of oxidation which NaOH increases the rate of oxidation is above this limit. We are therefore of the opinion that the injurious (etching?) effect of the NaOH is not directly related to the normal processes of oxidation, but that weak bases do not produce this injurious effect in concentrations in which they can be applied. It is the fact that in the fertilized eggs an increase in the rate of oxidations can apparently only be produced by the addition of weak bases.

The second, and perhaps more interesting, question is whether weak bases influence the rate of oxidation of the egg so powerfully while they are absolutely neutral. There are two possible explanations for this. One is that the bases raise the rate of oxidation indirectly by causing a liquefaction of the egg layer. This in itself causes, as was shown by the experiment, a rapid increase in the rate of oxidation.

through oxidations, and more rapidly by the weak bases than by the strong ones, since the weak bases diffuse more rapidly into the egg. Since the spermatozoon liquefies this substance also it becomes clear that the rate of oxidations in the fertilized egg is no longer affected by weak bases.

SUMMARY OF RESULTS.

1. It is shown that bases influence the rate of oxidations differently in the fertilized and unfertilized egg of *Strongylocentrotus purpuratus*.

2. Strong bases, like NaOH and $N(C_2H_5)_4OH$, accelerate the rate of oxidations in the fertilized egg of *S. purpuratus* only if their concentration is above $10^{-3}N$; this concentration suppresses the phenomena of development of the egg.

3. Weak bases, like NH_4OH and methylamine, accelerate the rate of oxidations in the fertilized egg only slightly; the reason being that the weak bases are too slightly dissociated to produce in the concentrations admissible in the experiment the injurious (etching?) effects which the highly dissociated bases produce in this case.

4. These facts do not warrant any conclusion concerning the localization of the normal processes of oxidations in the egg.

AN IMPORTANT CHEMICAL DIFFERENCE BETWEEN THE EGGS OF THE SEA URCHIN AND THOSE OF THE STAR-FISH.

By A. P. MATHEWS.

(From the Marine Biological Laboratory. Woods Hole.)

(Received for publication, April 21, 1913.)

The eggs of the sea urchin, *Arbacia punctulata*, differ markedly in their physiological properties from those of the star-fish, *Asterias forbesii*. The sea-urchin egg is remarkably stable, resistant to oxidation, has a very low rate of respiration and is not easily stimulated to artificial parthenogenesis; the star-fish egg, on the other hand, is, after maturation, very easily oxidized, has a rapid rate of respiration, forms sulphuretted hydrogen when mixed with sulphur, is easily destroyed by oxygen, easily liquefied by heat and is easily cytolysed by anesthetics. It is readily, even by shock, caused to develop parthenogenetically. Moreover after maturation a steady growth of the nucleus takes place, whereas in *Arbacia* the nucleus after maturation remains of a very small size.

Five or six years ago I found an important chemical difference between these eggs to be that cholesterol was lacking in the star-fish egg, but present in some quantity in that of the sea urchin. In view of the relation of cholesterol to hemolysis this observation offers a possible explanation of the great ease of cytolysis of the star-fish egg as compared with the sea-urchin.

The eggs were pressed from the ovary through cheese-cloth to remove the connective tissue, and the mass then extracted three times with a large amount of 95 per cent alcohol, boiling for one hour each time, and then once with boiling ether. The united extracts were evaporated on the water bath, the residue extracted with ether repeatedly, filtered from insoluble substances, and the ether poured into acetone. The fat and cholesterol remain in solution; the lecithin is precipitated. The acetone filtrate was evaporated to dryness, the oily residue saponified with alcoholic

sodium hydrate and, after the addition of sodium sulphate and some water, was shaken out with ether repeatedly. The ether was washed several times with sodium carbonate solution and evaporated to dryness. The residue was very small in amount, not crystalline; it looked like oleic acid. It gave no positive tests for cholesterol either by Salkowski's or the Liebermann-Burchard method. I have repeatedly sought for cholesterol in these eggs varying the procedure but I have never been able to find it. On one occasion when the ovaries were not ripe the fatty residue of the ether after repeated saponifications, both with alkali and acid, gave a very faint, transitory green such as cholesterol gives in the Liebermann test, and there may have been a very small amount of cholesterol present; but no crystals could be obtained. In view of the fact that the color reaction is probably not specific I am doubtful whether there was a trace of cholesterol present or not. It could not be positively identified. It may be mentioned that cholesterol in combination as in lanolin gives the Liebermann-Burchard reaction very strongly.

The same methods applied to the sea-urchin egg gave, as usual, a crystalline mass on evaporating the ether after saponification; the crystals looked like cholesterol and gave a typical reaction of Salkowski. I may say that the extract of the whole body of the star-fish contains cholesterol in abundance.

Another very interesting peculiarity of the star-fish egg is the character of its phosphatide. It resembles the jecorin described by Drechsel. A large quantity of eggs was extracted with hot alcohol and ether; the lecithin (?) precipitated from the ether solution in the usual way, redissolved in ether (not anhydrous) and reprecipitated with acetone and the process repeated until it dissolved quite clear in the ether and did not settle out a white substance when standing in the cold. This white substance coming out of the ether had a sweet taste, but had no reducing action on Fehling's solution either before or after heating with hydrochloric acid. The phosphatide thus prepared is more hygroscopic than lecithin from the brain or eggs. It makes unusually beautiful, regular myelin forms when shaken with water, and it seems to be toxic for sea-urchin eggs. It was probably not a pure substance. It contains a large amount of a reducing sugar which, calculated as glucose, amounts to 10.51 per cent by weight. The nature of this

sugar was not determined; an osazone was prepared; the fermentation test was indecisive. The lecithin itself does not reduce Fehling's solution but only after it has been heated with acid. I heated it for ten hours with 3.5 per cent HCl and determined the sugar by the reduction of Fehling's solution according to the method of Munson and Walker. This phosphatide also contains sulphuric acid in an ester form like Koch's sulphatide. It contained in a single analysis 1.19 per cent of sulphur in an oxidized form. This is in organic combination. The fatty acids are very largely oleic, or a similar acid, having an ether-soluble lead salt, The analysis of this impure phosphatide resulted as follows:

Glucose (?).....	10.51 per cent.
Fatty acids	46.16 per cent.
Phosphorus.....	3.57 per cent.
Sulphur	1.19 per cent.

Of the fatty acid approximately 71.35 per cent was recovered as oleic (?) acid. This phosphatide also contains a considerable amount of magnesium, but I did not determine it quantitatively.

I may mention that, of the total ether-soluble portion of the alcohol-ether extract of these eggs, the lecithin in one case weighed 0.6105 gram; the fat, the part not precipitated by acetone, 0.6055 gram; so that there are about equal quantities of fat and lecithin.

SUMMARY.

Cholesterol is either absent altogether or present in very small amount in the star-fish egg. It could not be positively found in the eggs of *Asterias forbesii*. It is present in considerable quantities in the sea-urchin egg. This difference possibly is correlated with the greater sensitiveness to cytolysis of the star-fish egg. The phosphatide of the star-fish contains about 10 per cent of a reducing sugar in firm combination and also sulphuric acid.

THE INFLUENCE OF HYPERTONIC SOLUTION UPON THE RATE OF OXIDATIONS IN FERTILIZED AND UNFERTILIZED EGGS.

BY JACQUES LOEB AND HARDOLPH WASTENEYS.

(From the Rockefeller Institute for Medical Research, New York.)

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I.

In a series of papers published since 1905 one of the writers has shown that in the egg of the sea urchin the initiation of normal development requires two different agencies. The one is needed to call forth a typical change in the surface of the egg which results in the formation of a more or less typical "fertilization membrane." This change causes the egg to segment and if the temperature is low some of these eggs may reach an early larval stage. At room temperature, however, the eggs begin to disintegrate after artificial membrane formation, as a rule, during the first cell division. These facts prove that the artificial membrane formation (*e.g.*, by butyric acid) suffices to set the whole machinery of cell division and development into motion but that the egg is sickly and disintegrates the more rapidly the higher the temperature.¹

It was shown by the same author that this disintegration after artificial membrane formation is retarded or suppressed if we deprive the egg of oxygen or if we retard the rate of oxidations in the egg through the addition of a trace of KCN. The addition of a sufficient amount of chloral hydrate (or probably any other narcotic) acts in the same way, although chloral hydrate does not lower the rate of oxidations. But all these agencies have one object in common, namely, that they inhibit the processes of nuclear and cell division. The disintegration of the unfertilized

¹ Loeb: *Die chemische Entwicklungserregung des tierischen Eies*, Berlin. 1909; *The Mechanistic Conception of Life*, 1912.

egg after membrane formation is therefore connected either with oxidations or with the nuclear and cell divisions consequent upon oxidations. We can cure the egg from this disease by one of two agencies: We either put it for a short time (from 30 to 60 minutes) into a neutral hypertonic solution; or we put it for a longer time (about 3 hours) into sea water which is free from oxygen or which contains some KCN. The former method gives more uniform results. Such eggs develop into larvae at room temperature.

These results gained in importance since it could be shown that the developmental effect of the spermatozoon is also due to two different agencies, one of which causes merely membrane formation while the other produces the corrective effect.²

Warburg had already found that the artificial membrane formation raises the rate of oxidations in the egg to the same height as the entrance of the spermatozoon, as was to be expected and as we were able to confirm.³ Since Loeb had found that the hypertonic solution acts only in the presence of free oxygen and that its action is suppressed by the addition of a trace of KCN, it was of interest to find out whether or not the hypertonic solution alters the rate of oxidations in the egg after artificial membrane formation. The experiments were made in this way, that the rate of oxidations in the eggs, after artificial membrane formation, was determined. first in normal sea water and later in hypertonic solution.

These experiments are by no means simple, since it is necessary that all the eggs possess membranes; for we shall see afterwards that in unfertilized eggs without membranes the hypertonic solution causes a decided rise in the rate of oxidations.

The unfertilized eggs of one female, *Strongylocentrotus purpuratus*, were divided into six equal parts. Two remained unaltered and served as checks. The eggs of two lots were treated with butyric acid and all formed membranes. One of these lots was put into normal sea water, the other into hypertonic sea water (50 cc. sea water + 8 cc. $\frac{5M}{4}$ NaCl) such as is used to cause the eggs to develop normally after artificial membrane formation. The fifth lot was fertilized with sperm. The sixth lot served for some other experiment which does not concern us here. The eggs remained in each solution one hour. Temperature 18°C.

² Loeb: *loc. cit.*

³ Warburg: *Zeitschr. f. physiol. Chem.*, lxvi, p. 305, 1910.

TABLE I.

	OXYGEN CONSUMED	COEFFICIENT OF OXIDATIONS
	<i>mgm.</i>	
1. Unfertilized eggs in normal sea water (control 1).....	0.12	1.00
2. Unfertilized eggs in normal sea water (control 2).....	0.12	1.00
3. Unfertilized eggs after membrane formation in normal sea water.....	0.53	4.40
4. Unfertilized eggs after membrane formation in hypertonic sea water.....	0.54	4.50
5. Fertilized eggs in normal sea water.....	0.57	4.70

It is obvious that the hypertonic solution does not increase the rate of oxidations in the unfertilized eggs after artificial membrane formation. Table II gives the result of a second experiment of the same kind. Temperature 15°C.

TABLE II.

	OXYGEN CONSUMED PER HOUR	COEFFICIENT OF OXIDATIONS
	<i>mgm.</i>	
1. Unfertilized eggs in normal sea water.....	0.18	1.00
2. Unfertilized eggs after membrane formation in normal sea water.....	0.85	4.72
3. Unfertilized eggs after membrane formation in hypertonic sea water.....	0.88	4.88
4. Fertilized eggs in normal sea water.....	0.82	4.55

The result is identical with that in table I. Membrane formation raises the rate of oxidations to the same height as fertilization, but the subsequent treatment of these eggs with the hypertonic solution has no effect upon the rate of oxidations. This experiment was repeated three times with the same results as shown in Table III. The oxygen consumption is always for one hour at 18°C.

All these experiments prove conclusively that the curative effect of the hypertonic solution after the artificial membrane formation is not due to an increase in the rate of oxidations in the egg. It cannot be said, however, that it is independent of oxidations,

TABLE III.

NUMBER OF EXPERIMENT		OXYGEN CONSUMED PER HOUR	COEFFICIENT OF OXIDATIONS
		mgm.	
1	Unfertilized eggs after membrane formation in normal sea water....	0.83	1.00
	Unfertilized eggs after membrane formation in hypertonic sea water....	0.74	0.90
2	Unfertilized eggs after membrane formation in normal sea water....	0.52	1.00
	Unfertilized eggs after membrane formation in hypertonic sea water....	0.54	1.04
3	Unfertilized eggs after membrane formation in normal sea water....	0.74	1.00
	Unfertilized eggs after membrane formation in hypertonic sea water....	0.70	0.90

since the curative effect of the hypertonic solution is retarded or suppressed if the oxidations in the egg are suppressed. Loeb formerly suggested that through the exposure to the hypertonic solution an oxidation product is formed in the egg whereby the latter is saved from the threatening disintegration. It may be that an injurious substance contained in the egg after membrane formation is destroyed or that a new substance lacking in the egg is supplied. The same curative effect can be produced more slowly through other processes in the egg which take place in the absence of oxygen.

II. THE INFLUENCE OF HYPERTONIC SOLUTIONS UPON THE RATE OF OXIDATIONS IN FERTILIZED EGGS.

Since the unfertilized egg after artificial membrane formation behaves in regard to oxidations like a fertilized egg, it was of interest to find out whether or not the hypertonic solution accelerates the rate of oxidations in eggs fertilized by sperm. O. Warburg states that this is the case, and that the increase may be 300 per cent.⁴ We absolutely failed to notice any increase in the rate of oxidations when the eggs of *S. purpuratus* were put into hypertonic sea water, no matter how great the degree of hypertonicity. In

⁴ Warburg: *Zeitschr. f. physiol. Chem.* 1x, p. 442, 1909.

part of the experiments, 1-3, the rate of oxidations was successively measured in the same eggs for one and a half hours in normal sea water, and then for one hour in the hypertonic sea water. In the experiments 4-7 the eggs of the same female were divided into eight equal parts; four of these were put into normal sea water, the others into hypertonic sea water of different concentrations. This experiment may incidentally also serve as a check for the accuracy of the method.

The temperature was 18°C. and the consumption of oxygen measured for one and a half hours. Table IV gives the results.

There can be no doubt about the fact that the hypertonic solution does not increase the rate of oxidations in the fertilized egg of *Strongylocentrotus purpuratus*. This harmonizes with our previous

TABLE IV.

NUMBER OF EXPERIMENT		OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
		mgm.	
1	Fertilized eggs in normal sea water.	0.87	1.00
	Fertilized eggs in 50 cc. normal sea water + 8 cc. $\frac{5M}{2}$ NaCl.....	0.86	0.99
2	Fertilized eggs in normal sea water.	0.60	1.00
	Fertilized eggs in 50 cc. normal sea water + 8 cc. $\frac{5M}{2}$ NaCl.....	0.52	0.87
3	Fertilized eggs in normal sea water.	0.55	1.00
	Fertilized eggs in 50 cc. normal sea water + 8 cc. $\frac{5M}{2}$ NaCl.....	0.59	1.07
4	Fertilized eggs in normal sea water.	1.30	1.00
	Fertilized eggs in 50 cc. normal sea water + 4 cc. $\frac{5M}{2}$ NaCl.....	1.27	0.98
5	Fertilized eggs in normal sea water.	1.30	1.00
	Fertilized eggs in 50 cc. normal sea water + 12 cc. $\frac{5M}{2}$ NaCl.....	1.54	1.20
6	Fertilized eggs in normal sea water.	1.33	1.00
	Fertilized eggs in 50 cc. normal sea water + 12 cc. $\frac{5M}{2}$ NaCl.....	1.53	1.20
7	Fertilized eggs in normal sea water.	1.33	1.00
	Fertilized eggs in 50 cc. normal sea water + 16 cc. $\frac{5M}{2}$ NaCl.....	1.57	1.20

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result that the hypertonic solution does not increase the rate of oxidations in unfertilized eggs of the same species after artificial membrane formation, since the membrane formation is the essential feature in the causation of development by sperm or by artificial means.

III.

The fact that a hypertonic solution does not increase the rate of oxidations in the unfertilized eggs after artificial membrane formation seems at first sight to contradict an observation made by Warburg at Naples, that the hypertonic solution raised the rate of oxidations in unfertilized eggs (which have not been submitted to the process of membrane formation). Warburg found that the hypertonic solution raises the rate of oxidations in such eggs as much as ten times.⁵ We repeated these experiments on the eggs of *Strongylocentrotus purpuratus* at Pacific Grove, and were able to confirm Warburg's results although the rise in the rate of oxidations was much smaller than that observed in his experiments. In our experiments the rate of oxidations was first determined for one and a half hours in normal sea water and then for one and a half hours in hypertonic sea water. Table V on the following page gives the results. The temperature was 18°C.

It should be pointed out that experiments 2 and 3, and 4 and 5, are made on equal lots of eggs. The results may serve as a check for the accuracy of the method.

The question arises: Why is it that the hypertonic solution causes a rise in the rate of oxidations in the unfertilized egg without membrane formation, while it has no such effect on the same eggs after membrane formation? The answer is that the hypertonic solution can cause the membrane formation and that it only raises the rate of oxidations in those eggs in which it causes membrane formation. In Loeb's original method of causing artificial parthenogenesis by merely putting the eggs into a hypertonic solution, the hypertonic solution had two kinds of effects: it caused first the membrane formation (or the formation of a gelatinous surface film) and at the same time furnished the curative effect.

⁵ Warburg: *Zeitschr. f. physiol. Chem.*, lx, p. 443, 1909.

TABLE V.

NUMBER OF EXPERIMENT	UNFERTILIZED EGGS WITHOUT MEMBRANES IN	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
		<i>mgm.</i>	
1	Normal sea water.....	0.45	1.00
	Hypertonic (50 cc. sea water + 4 cc. $\frac{5M}{2}$ NaCl).....	0.65	1.40
2	Normal sea water.....	0.50	1.00
	Hypertonic (50 cc. sea water + 6 cc. $\frac{5M}{2}$ NaCl).....	0.92	1.80
3	Normal sea water.....	0.49	1.00
	Hypertonic (50 cc. sea water + 6 cc. $\frac{5M}{2}$ NaCl).....	0.84	1.70
4	Normal sea water.....	0.33	1.00
	Hypertonic (50 cc. sea water + 6 cc. $\frac{5M}{2}$ NaCl).....	0.73	2.20
5	Normal sea water.....	0.35	1.00
	Hypertonic (50 cc. sea water + 8 cc. $\frac{5M}{2}$ NaCl).....	0.73	2.50
6	Normal sea water.....	0.46	1.00
	Hypertonic (50 cc. sea water + 8 cc. $\frac{5M}{2}$ NaCl).....	1.19	2.60
7	Normal sea water.....	0.48	1.00
	Hypertonic (50 cc. sea water + 9 cc. $\frac{5M}{2}$ NaCl).....	1.23	2.60
8	Normal sea water.....	0.29	1.00
	Hypertonic (50 cc. sea water + 9 cc. $\frac{5M}{2}$ NaCl).....	0.90	3.10
9	Normal sea water.....	0.30	1.00
	Hypertonic (50 cc. sea water + 9 cc. $\frac{5M}{2}$ NaCl).....	0.67	2.20
10	Normal sea water.....	0.56	1.00
	Hypertonic (50 cc. sea water + 12 cc. $\frac{5M}{2}$ NaCl).....	1.29	2.30
11	Normal sea water.....	0.38	1.00
	Hypertonic (50 cc. sea water + 16 cc. $\frac{5M}{2}$ NaCl).....	0.97	2.60

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That the hypertonic solution can cause a cytolytic effect upon the cortical layer of the egg was shown by experiments on the eggs of *Lottia* (a mollusc) whose chorion can be liquefied by hypertonic solutions as well as by bases, if free oxygen is present.⁶

That the two agencies, the membrane-forming and the corrective one, can act simultaneously is not surprising. The same fact can be shown for bases. It is immaterial whether the base is applied first and is then followed by the corrective action of the hypertonic solution or whether both agencies are applied simultaneously.

In our experiments the effect of the hypertonic solution upon oxidations was smaller than that caused by membrane formation with butyric acid. In the latter case, the rate of oxidations was raised from four to six times, while the hypertonic solution, when acting upon unfertilized eggs without membrane formation, raised the rate as a rule to not more than two and one-half and at the utmost three times the amount observed in the same eggs in normal sea water. This difference can be accounted for either by the fact that an exposure of one and a half hours of the eggs of *S. purpuratus* to hypertonic sea water as a rule does not suffice to bring about membrane formation and development; or by the fact that these experiments were made early in the season when fertilization did not raise the rate of oxidations as much as it did later in the season.

We repeated these experiments later in the season to find out whether the rate of oxidations would increase if the eggs remained a longer time in the hypertonic sea water. The temperature was 18°C. The consumption of oxygen was determined for each hour.

TABLE VI.

UNFERTILIZED EGGS IN	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm.	
Normal sea water.....	0.16	1.00
Hypertonic sea water; 1st hour.....	0.67	4.18
Hypertonic sea water; 2d hour.....	0.79	4.94
Hypertonic sea water; 3d hour.....	0.64	4.00
Hypertonic sea water; 4th hour.....	0.56	3.55
Hypertonic sea water; 5th hour.....	0.51	3.18

⁶ Loeb: *Univ. of Calif. Publ. Physiol.*, iii, 1905.

A repetition of the experiment gave a similar result. In this case the hypertonic solution had the same effect upon the rate of oxidations as fertilization or membrane formation. It may also be that by chance we are dealing in this case with the eggs of a female which were very susceptible to the treatment with hypertonic solution while in the previous experiments the eggs had been more or less refractory. As we stated, not the eggs of every female of *purpuratus* develop if treated with hypertonic sea water.

These results support also the idea that the rise in the rate of oxidations is due to the membrane-forming action of the hypertonic solution, which of course takes place during the first two hours. We see that the maximal increase in the rate of oxidations takes place during that time.

IV.

If it is true that the increase in the rate of oxidations observed in these cases is merely due to the membrane-forming effect of the hypertonic solution, we should expect that if we add a weak base to the hypertonic solution, the increase in the rate of oxidations should be no greater than that caused by the weak base alone, the reason being that the weak base alone causes the membrane formation.⁷ This reasoning is supported by facts. The rate of oxidations was compared in unfertilized eggs (without membranes) in alkaline sea water and hypertonic sea water to which the same amount of base had been added. The hypertonic sea water consisted of 50 cc. sea water + 8 cc. $\frac{5M}{2}$ NaCl + KCl + CaCl₂. The eggs were distributed into equal portions. One-half was put into 50 cc. of sea water + 1 cc. of the base; the other half of the eggs was first put into normal sea water and then into 50 cc. of hypertonic sea water + 1 cc. of the same base. Time of exposure one and a half hours; temperature 18°C.

⁷ Loeb: *Journ. of Exp. Zoölogy*, xiii, p. 577, 1912.

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TABLE VII.

NUMBER OF EXPERIMENT	UNFERTILIZED EGGS (WITHOUT MEM- BRANES) IN	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
		mgm.	
1	Normal sea water.....	0.22	1.00
	50 cc. hypertonic sea water + 1 cc. $\frac{N}{10}$ NH_4OH	1.20	5.40
	50 cc. normal sea water + 1 cc. $\frac{N}{10}$ NH_4OH	0.88	4.00
		0.37	1.00
2	Normal sea water.....	0.37	1.00
	50 cc. hypertonic sea water + 1 cc. $\frac{N}{10}$ benzylamine	1.89	5.10
	50 cc. normal sea water + 1 cc. $\frac{N}{10}$ benzylamine.....	1.75	4.70
		0.36	1.00
3	Normal sea water.....	0.36	1.00
	50 cc. hypertonic sea water + 1 cc. $\frac{N}{10}$ butylamine	1.73	4.80
	50 cc. normal sea water + 1 cc. $\frac{N}{10}$ butylamine.....	1.67	4.60

It is obvious that the weak base alone raised the rate of oxidations practically to the same height as the combination of base and hypertonic sea water. The whole rise was due to the membrane-forming effect for which the weak base was sufficient.

In the case of a strong base, the result may be different, since neither the base nor the hypertonic solution alone may cause membrane formation (or the change in the cortical layer of the egg) necessary for development. The following may serve as an example. Duration of experiment one and a half hours; temperature 18°C.

TABLE VIII.

UNFERTILIZED EGGS (WITHOUT MEMBRANE FORMATION) IN	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm.	
Normal sea water.....	0.41	1.00
50 cc. hypertonic sea water + 1 cc. $\frac{N}{10}$ NaOH	0.81	2.00
50 cc. normal sea water + 1 cc. $\frac{N}{10}$ NaOH	0.46	1.20

In this case the NaOH had little effect and hence the hypertonicity caused a noticeable increase in the rate since it probably increased the number of eggs in which the process of membrane formation was started.

v.

Finally, we wish to report an experiment which does not strictly belong here but which shows that the mere cytolysis of the cortical layer is responsible for the increase in the rate of oxidations observed after membrane formation either by a spermatozoon or by butyric acid. It is possible to cause complete cytolysis of the unfertilized egg of *S. purpuratus* with saponin and we found that this increases the rate of oxidations to the same extent as fertilization by sperm. The following may serve as an example. Unfertilized eggs of *S. purpuratus* were used; temperature 15°C.

TABLE IX.

	OXYGEN CONSUMED PER HOUR	COEFFICIENT OF RATE OF OXIDATIONS
	<i>mgm.</i>	
Unfertilized eggs	0.15	1.00
The same eggs after cytolysis with saponin	1.07	7.10
Unfertilized eggs	0.22	1.00
The same eggs after cytolysis with saponin	0.80	3.60

The variation in the effect of cytolysis in the two experiments may be due to the fact that in the second experiment an excessive amount of saponin was used.

This experiment proves that the increase in the rate of oxidations due to fertilization or artificial membrane formation is merely caused by the cytolysis of the cortical layer.

VI. THEORETICAL REMARKS.

It seems that all the experiments point very clearly towards one conclusion, namely, that the hypertonic solution raises the rate of oxidations in the unfertilized eggs of *S. purpuratus* only under one condition, *i.e.*, if it causes the change at the surface of the egg underlying membrane formation. In eggs which have undergone the process of membrane formation either by fertilization or by a treatment with butyric acid, the hypertonic solution causes no further increase in the rate of oxidations.

SUMMARY.

1. The unfertilized eggs of sea urchins which have undergone artificial membrane formation die if not treated with a hypertonic solution. It is shown in these experiments that the rate of oxidations in such eggs is not increased by the hypertonic solution.

2. It is shown that the hypertonic solution does not cause an increase in the rate of oxidations of fertilized eggs of *Strongylocentrotus purpuratus*.

3. Hypertonic solutions increase the rate of oxidations in unfertilized eggs which have not undergone the process of membrane formation, as Warburg observed. This increase is purely due to the fact that the hypertonic solution induces the change in the cortical layer of the egg which leads to membrane formation.

4. This conclusion is supported by the fact shown in this paper, that the addition of a weak base to normal sea water, which causes development (or membrane formation) increases the rate of oxidations in unfertilized eggs to the same amount as if it were added to hypertonic sea water. Since in this case the membrane-forming effect can be produced by the base alone the addition of the hypertonic solution can add nothing to the effect.

5. Complete cytolysis of the unfertilized egg by saponin raises the rate of oxidations to the same height as fertilization, thus showing that the cytolysis of the cortical layer of the egg is the essential feature in fertilization.

DO GLIADIN AND ZEIN YIELD LYSINE ON HYDROLYSIS?

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GLIADIN.

Kossel and Kutscher² failed to find any lysine in the alcohol-soluble proteins of maize or wheat flour, and their experience has been confirmed by others.³ It has since been generally assumed that alcohol-soluble proteins lack the lysine complex, and consequently gliadin and zein have been used in experiments, from the outcome of which conclusions have been drawn respecting the part played by lysine in nutrition, especially in connection with the synthesis of amino-acids by the animal organism.⁴ Observations have been recorded, however, which have shown that preparations of gliadin may sometimes yield lysine. Thus Abderhalden and Funk⁵ in discussing Henriques' experiments state that unless gliadin preparations are very carefully purified they always yield small amounts of lysine. They do not, however, say how they detected the lysine nor how the preparations should be purified.

Van Slyke⁶ by aid of his method for determining the distribution of nitrogen in proteins found in gliadin a quantity of nitrogen

¹ The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

² Kossel and Kutscher: *Zeitschr. f. physiol. Chem.*, xxxi, p. 165, 1900.

³ Abderhalden and Samuely: *ibid.*, xlv, p. 276, 1905; Osborne and Clapp: *Amer. Journ. of Physiol.*, xvii, p. 231, 1906; Ackermann: *Zeitschr. f. physiol. Chem.*, lxiv, p. 91, 1910.

⁴ Michaud: *Zeitschr. f. physiol. Chem.*, lix, p. 405, 1909; Henriques: *ibid.*, lx, p. 105, 1909; Osborne and Mendel: *this Journal*, xii, p. 473, 1912.

⁵ Abderhalden and Funk: *Zeitschr. f. physiol. Chem.*, lx, p. 418, 1909.

⁶ Van Slyke: *this Journal*, x, p. 15, 1911.

in the lysine fraction corresponding to 0.75 per cent of lysine. He considers this small amount to fall within the limit of experimental error and that it is not to be regarded as contradictory to the negative results of previous authors. Kossel and Gawrilow⁷ found that 0.8-0.4 per cent of the total nitrogen of hordein, equal to about 2 per cent of lysine, reacts with formaldehyde, whereas none of the nitrogen of zein reacts therewith. This observation shows that hordein may contain a small proportion of lysine, for there is reason to believe that lysine furnishes the only free amino groups present in most proteins.

In a recent paper⁸ from this laboratory attention was called to the fact that a preparation of gliadin, which had been made with care, and was supposedly as pure as preparations of this protein can reasonably be made, yielded a very small amount of lysine when hydrolyzed by long boiling with sulphuric acid. The question was then left open whether this lysine originated from a contamination of the preparation or from the gliadin itself.

Since it has become a matter of importance to know whether or not gliadin prepared by the usual methods is wholly free from lysine we have subjected the preparation previously examined to a rigid fractional precipitation from alcoholic solution, and have again detected lysine in each of the extreme fractions, and in approximately the same proportion as that previously found in the original preparation. No evidence was secured which indicated the presence of glutenin, or any other protein in this preparation, and as the same relatively very high proportion of nitrogen as ammonia, and low proportion of basic nitrogen, which characterize gliadin, were found in the two extreme fractions and in the original preparation we are inclined to think that this preparation contained no other protein than gliadin, and that gliadin does in fact yield so little lysine that its presence in purified preparations has heretofore escaped detection by the usual methods of analysis. The amount of lysine in the original preparation was so small that in following Kossel's method no precipitate was found when the alcoholic solution of picric acid was added to the properly concentrated alcoholic solution of the amino-acids pre-

⁷ Kossel and Gawrilow: *Zeitschr. f. physiol. Chem.*, lxxxi, p. 274, 1912.

⁸ Osborne and Mendel: *this Journal*, xii, p. 473, 1912.

precipitated by phosphotungstic acid, after the arginine and histidine had been precipitated by silver nitrate and baryta.

By allowing the alcoholic solution containing the picric acid to evaporate slowly a semi-crystalline residue remained which was extracted with alcohol. The part which was not dissolved was recrystallized from water, yielding a product which appeared in all respects like lysine picrate. This darkened on heating at about 225° and exploded at 256° in the manner characteristic of lysine picrate. When mixed with pure lysine picrate the behavior on heating was unchanged. The nitrogen content found by digesting with sulphuric and salicylic acids and zinc dust, and then boiling for several hours, was 18.34 per cent. Calculated for lysine picrate, 18.68 per cent.

Since large quantities of alcohol were used in extracting the wheat gluten, from which this gliadin was obtained, it was possible that the lysine thus found came from a contamination with glutenin or some other protein present in the original gluten. If this were so the greater part of such contamination should appear as an insoluble residue when the preparation was dissolved again in a limited quantity of 70 per cent alcohol. We accordingly treated 500 grams of the gliadin with 2500 cc. of 70 per cent (by volume) alcohol and obtained a slightly turbid solution which on long standing deposited a very small amount of insoluble matter. By repeatedly washing this sediment by decantation with 70 per cent alcohol only an insignificant quantity remained, from which we conclude that this preparation of gliadin contained, at the most, but traces of glutenin, or other protein insoluble in dilute alcohol.

The somewhat opalescent alcoholic solution deposited nothing more on standing or centrifugalization, and became perfectly clear on warming slightly. In order to find whether or not this solution could be separated by fractional precipitation into products indicating the presence of two or more proteins, of which one might contain the lysine, we treated the solution as follows:

The entire solution, of 3 liters' volume, was mixed with 3 liters of 80 per cent alcohol, by pouring the two simultaneously into a large jar, and stirring rapidly during the mixing, so as to avoid precipitating any gliadin by locally raising the concentration of the alcohol above 75 per cent through imperfect mixing. A turbid solution resulted which showed no sign of a precipitate. This

solution was then mixed, in the same way, with 6 liters of 85 per cent alcohol, whereupon a flocculent precipitate separated from the solution, now containing 80 per cent of alcohol. After stirring violently the precipitate separated in flocks and soon settled, leaving the solution nearly clear. In this way any fraction less soluble in the more concentrated alcohol should be separated from that soluble therein, for the increased volume of the stronger alcohol could be expected to keep the latter in solution.

After the precipitate had settled it became coherent, whereupon the nearly clear solution was decanted very completely. The precipitate was then treated with about 1500 cc. of 80 per cent alcohol, by which little of it was dissolved. It was thereby converted into a voluminous, snow-white, flocculent substance which differed in its physical character from the gliadin precipitates usually obtained by pouring gliadin solutions into strong alcohol. This was separated from the 80 per cent alcoholic washings by centrifugalization, suspended in 80 per cent alcohol and then left for about forty hours to settle. The colloidal suspension which remained was treated with a few drops of a solution of ammonium acetate, dissolved in 80 per cent alcohol, which caused a practically complete separation, leaving the alcohol nearly clear, and rendered the precipitate glutinous. The precipitate was then extracted with 70 per cent alcohol, the undissolved residue centrifugated out, dehydrated by absolute alcohol, and dried over sulphuric acid. This product, A, which weighed 55 grams, should contain whatever glutenin was present in the original preparation, any gliadin that had been altered and rendered less soluble by the processes employed in making the original preparation, any contaminating protein sparingly soluble in strong alcohol, or, if gliadin is a mixture of proteins, as some have supposed, more or less of any constituent of such a mixture which was less soluble in stronger alcohol than the rest.

The 12 liters of 80 per cent alcohol, from which A had been separated, after standing several days, gradually yielded a small, semi-fluid, transparent deposit from which the solution was decanted. This deposit was dissolved completely and readily by cold 70 per cent alcohol, its solution united with that obtained by digesting A with 70 per cent alcohol, concentrated to a syrup on a steam bath, and then poured into a large volume of absolute

alcohol. The resulting precipitate, after digesting with absolute alcohol and ether, was freed from the latter in a desiccator over sulphuric acid. This fraction, *B*, weighed 38 grams.

The 12 liters of 80 per cent alcoholic solution, together with the washings of the same strength of alcohol, 3 liters, were united, concentrated in a vacuum to about 1500 cc. and then on a steam bath to a syrup which was finally poured into a large volume of absolute alcohol. The precipitate thus produced was dehydrated by digesting with absolute alcohol, washed with ether, and freed from alcohol and ether in a desiccator over sulphuric acid. This fraction, *C*, weighed 298 grams.

The original gliadin preparation was thus divided into three fractions; *A*, insoluble in 70 per cent alcohol; *B*, insoluble in 80 per cent alcohol but soluble in 70 per cent; and *C*, soluble in 80 per cent alcohol.

In order to detect differences between the extreme fractions *A* and *C*, the following analytical data were secured which are given as per cent of the protein.

PREPARATION		NH ₂ N	BASIC N	ARGININE	HISTIDINE	LYSINE	TYROSINE
<i>A</i>	I	4.66	0.76	2.48	1.43	0.15	1.39
	II	4.66	0.76	2.43	1.44		
<i>C</i>	I	4.39	1.01	2.92	1.49	0.07	1.50
	II	4.39	1.01	2.90	1.48		
Original	I			2.67	1.63	0.16	1.54
	II			2.78	1.49		1.68

From fractions *A* and *C* lysine was isolated in the same way as from the original preparation. No precipitate of lysine picrate was obtained by adding an alcoholic solution of picric acid to the suitably concentrated alcoholic solution of the amino-acids precipitated by phosphotungstic acid, and it was only by persistent effort that any lysine picrate was obtained from either solution. The identity of the picrate was established by the decomposition point, which was unchanged on mixing with pure lysine picrate, and also by the nitrogen content.

If the probable limits of accuracy of the methods employed in making the above determinations are considered, the results

obtained afford no basis for concluding that the original preparation has been separated into two chemically different parts. The only difference worthy of consideration is that between the ammonia nitrogen of *A* and *C*. This difference is distinctly beyond the limits of the analytical method, but it must be recalled that a little ammonium acetate solution was used to cause the colloidal suspension of *A* to separate from the final 80 per cent alcoholic wash solution. It is consequently not improbable that some ammonia was combined with, or adsorbed by, the protein and thus contributed to the higher result of this determination.

It is thus evident that the lysine found in the original preparation did not come from a contamination with glutenin, for this protein contains less ammonia nitrogen, and more basic nitrogen and also more arginine than either of the fractions *A* or *C*.

It is also to be noted that lysine was found in both *A* and *C* and since no importance can be attached to the small difference between the quantities found we have no evidence that the original preparation consisted of a lysine-yielding and a lysine-free protein. The fact that the original preparation of gliadin was separated into three fractions which differed in solubility in alcohol of various strengths might be considered as evidence of the presence of more than one protein in the original preparation. Such evidence, however, has no force, for, in the process of isolation, nearly all proteins are liable to slight alterations whereby more or less of them is converted into insoluble products. Furthermore, preparations of proteins made by the only methods now available consist to a greater or less extent of salts of these proteins which differ in solubility from the free protein, as well as from one another. Such differences as here appear cannot, therefore, be accepted as good evidence of the presence of more than one protein in the original preparation of gliadin.

The discovery of this small proportion of lysine in these preparations of gliadin shows how cautious we must be in concluding that any amino-acid is wholly lacking among the products of hydrolysis of a protein when the only evidence for such a conclusion is based on the failure to isolate it by direct crystallization either in the free state or as a crystalline compound with an added reagent.

ZEIN.

Having thus found lysine in a supposedly pure preparation of gliadin we made a careful examination of zein employing the same method for obtaining traces of lysine picrate which had proved successful with gliadin. Our long-continued and persistent efforts resulted only in crystalline picrates which decomposed at 272° and proved to be sodium picrate. No trace of lysine picrate was found. The sodium was derived from traces of this base in the large quantities of phosphotungstic acid and baryta used in the analysis.

THE INFLUENCE OF FEVER ON THE ELIMINATION OF CREATININE.

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It has long been recognized that the rate of body metabolism is intensified in the various febrile diseases, resulting in an increased elimination of the nitrogenous waste products in the urine. The study of the urine under these conditions has been of considerable service in the interpretation of intermediary processes which take place in the body during this disturbed state. Metabolism in fever, especially in typhoid fever and pneumonia, has recently received extensive consideration at the hands of a number of investigators, viz., van Hoogenhuyze and Verploegh,¹ Klercker,² Ewing and Wolf,³ Shaffer and Coleman,⁴ and Wolf and Lambert.⁵

That the elimination of total nitrogen and of its chief constituent, urea nitrogen, paralleled the rise in body temperature in various acute infections was noted long ago. The excretion of ammonia was found to be increased by some of the older investigators, though its excretion as determined with more reliable methods would indicate that the ammonia is not ordinarily greatly increased in fever, and further, that acidosis is not a prominent feature of this condition. Uric acid has quite generally been found to be increased during the height of fever, followed usually by a decline with the fall in temperature. Fever was likewise claimed by the older observers to increase the elimination of creatinine, though

¹ van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 161, 1908.

² Klercker: *Zeitschr. f. klin. Med.*, lxxviii, p. 22, 1909.

³ Ewing and Wolf: *Arch. of Int. Med.*, iv, p. 330, 1909.

⁴ Shaffer and Coleman: *ibid.*, iv, p. 538, 1909.

⁵ Wolf and Lambert: *ibid.*, v, p. 406, 1910.

the data on this point can hardly be regarded as conclusive, owing to the unreliability of the methods available.

Since Folin⁶ with his colorimetric method first demonstrated the absolute constancy in the elimination of creatinine by normal individuals under a variety of dietary conditions, this nitrogenous urinary constituent, which stands next to urea in point of quantity, has been a subject of study at the hands of numerous investigators. A lowered creatinine output has been observed to be associated with a variety of diseases,⁷ but the only condition which has been found to be accompanied by an immediate change in the elimination of creatinine is fever; and here the excretion of this constituent is increased, the amount of the increase apparently bearing a close relationship to the rise in body temperature. van Hoogenhuyze and Verploegh⁸ claim to have observed a slightly increased elimination of creatinine after stimulation with syrup of cola and strychnine, and a slight decrease after potassium bromide and absolute rest. The changes they observed were very slight and in no way comparable to the greatly increased elimination during fever. In view of the lack of knowledge with regard to the origin of creatinine, the increased excretion brought about by fever is of particular interest.

This rise in creatinine elimination during fever appears to have been first observed by Leathes⁹ after the production of a hyperpyrexia in normal subjects by the use of antityphoid vaccine. He found that the curve of the creatinine elimination followed both the temperature and the total nitrogen, but that the percentage of creatinine nitrogen in terms of total nitrogen fell somewhat below the normal percentage with a marked increase in the elimination of total nitrogen, though the absolute amount of the creatinine excretion was increased about 25 per cent at the highest point. Subsequently, van Hoogenhuyze and Verploegh¹⁰ noted that the maximum creatinine excretion appeared at the same time or within a few hours of the highest temperature. The observations of Klercker¹¹ on the elimination of creatinine and creatine in febrile

⁶ Folin: *Amer. Journ. of Physiol.*, xiii, p. 66, 1905.

⁷ Cf. Myers: *Amer. Journ. of Med. Sci.*, cxxxix, p. 256, 1910.

⁸ *Loc. cit.*

⁹ Leathes: *Journ. of Physiol.*, xxxv, p. 205, 1907.

¹⁰ *Loc. cit.*

¹¹ *Loc. cit.*

diseases, but more especially the contributions of Ewing and Wolf¹² and Shaffer and Coleman¹³ on protein metabolism during typhoid fever, and Wolf and Lambert¹⁴ on protein metabolism in pneumonia, have greatly amplified our knowledge on this subject. The increased excretion of creatinine during fever has likewise been noted by Manuchin,¹⁵ though the deductions he draws would hardly appear in accord with other observations.

Not only has an increased excretion of creatinine been observed during the active stages of fever, but a decrease in its elimination during convalescence. It has further been noted that sooner or later during the course of a fever creatine appears in the urine, and its elimination in increased quantities seems of grave diagnostic importance. This was strikingly shown in the series of fatal pneumonias reported by Wolf and Lambert. In general, a parallel between the febrile temperature, total nitrogen, urea and creatinine has been observed. Shaffer and Coleman found that the increased excretion of creatinine in fevers was hardly in proportion to the amount of body protein catabolized, their highest increase in the creatinine excretion being calculated as about 20 per cent. The destruction of body proteins during fever, though perhaps more severe, is quite comparable to that occurring during fasting, and Mendel and Rose,¹⁶ and Myers and Fine¹⁷ have observed that the total creatinine nitrogen (from both creatinine and creatine) forms a uniform proportion of the total nitrogen in starving rabbits.

In producing this increased metabolism in fever, as shown by the increased excretion of nitrogenous waste products and also by the increased elimination of carbon dioxide, several factors play a part, viz., the hyperthermia *per se*, the accompanying inanition, and the toxic agent causing the fever.

That pyrexia, produced by artificially raising the body temperature with a hot bath, or otherwise, will raise the level of body metabolism has been demonstrated. The carbon-dioxide elimination has been observed to be increased from 30 to 40 per cent under these conditions, and somewhat similar figures have been obtained

¹² *Loc. cit.*

¹³ *Loc. cit.*

¹⁴ *Loc. cit.*

¹⁵ Manuchin: *Russki Wratsch*, ix, pp. 18, 55, 89, 1910.

¹⁶ Mendel and Rose: this *Journal*, x, p. 213, 1911.

¹⁷ Myers and Fine: unpublished data.

for the nitrogen elimination. Linser and Schmid¹⁸ have pointed out that in man it is not until the body temperature is increased above 39°C. that there is an increase in protein metabolism. From this Krehl¹⁹ concludes that in infective fevers with a temperature under 40°C. the additional protein metabolism must be of toxic origin.

In infective fevers, whether in man or animals, it has often been noted that the rise in nitrogenous metabolism is greater than should be observed if due entirely to the pyrexia and accompanying inanition. This added excess in the nitrogen elimination has been explained as toxic in origin. Ewing and Wolf in their study of protein metabolism in typhoid fever observed that the highest excretion of nitrogen in proportion to body weight occurred in the so-called toxic cases and that, although this was generally coincident with a high temperature, the total nitrogen appeared to be less affected by the fever than by the intoxication. Some of the most widely quoted experiments on fever are those by May²⁰ performed on rabbits inoculated probably with the same organism as in our experiments. The inoculations were made during a period of starvation, and on the second day after inoculation the output of nitrogen exceeded the normal by 28.4 to 51.9 per cent. The rise in nitrogen elimination is quite comparable to that observed in our experiments below except that here the animals were not starving and the fever appeared several days later. These figures probably represent in a general way the increase in nitrogenous metabolism which may be observed in febrile conditions in man. The particular advantage of such experiments, as those of May on animals, is the fact that one has a fairly adequate control period which cannot be so readily obtained in similar diseases in man.

One other phase of the question, which is of the utmost practical importance in the treatment of fever in man, especially typhoid fever as shown by Shaffer and Coleman, is the diet. The lower specific dynamic action of carbohydrate in comparison to protein and the protective action of carbohydrate on protein is well known. May called attention, in his experiments in 1894, to the protective action of carbohydrate on body protein. By the use of diets high

¹⁸ Linser and Schmid: *Deutsch. Arch. f. klin. Med.*, lxxix, p. 514, 1904.

¹⁹ Krehl: *Clinical Pathology*, Amer. Edit. by Hewlett, 1907, p. 406.

²⁰ May: *Zeitschr. f. Biol.*, xxx, p. 1, 1894.

in caloric value and rich in carbohydrate, Shaffer and Coleman²¹ have shown it possible to retard and even prevent the febrile loss of body protein nitrogen in subjects of typhoid fever. This is of particular interest in this connection in view of the inhibitory influence of carbohydrate upon the excretion of creatine by starving animals.

EXPERIMENTAL PART.

Nine experiments are reported on rabbits inoculated with *Bacillus suipesticus*²² and three experiments upon animals whose temperature was raised artificially. The routine procedures in the first series of experiments consisted in determinations of total nitrogen, urea, ammonia, creatinine, creatine, chlorides, phosphates, potassium, and the routine clinical examination of the urine, together with morning and evening temperature observations during the fever period, and a previous control period of four or more days. In the last three experiments determinations of total nitrogen, creatinine, creatine and chlorides were made.

The analytical methods employed were: Kjeldahl method for total nitrogen, Benedict method for urea,²³ Folin methods for ammonia and creatinine,²⁴ Benedict-Myers modification of the Folin method for creatine,²⁵ Volhard-Harvey²⁶ method for chlorides, titration with uranium nitrate for phosphates and Drushel's method for potassium.²⁷

The rabbits were all healthy animals, females being used because it is somewhat easier to compress the bladder. Prior to beginning the experiment, the animals were placed upon a uniform carrot diet for a period of several days. During the experiment the bladder was squeezed out at a definite time each morning, the animal weighed, and then given its daily ration which was ordinarily consumed during the day in the control period. After inoculation or placing in an incubator the appetite of the animals declined, obvi-

²¹ *Loc cit.*; also Coleman: *Amer. Journ. of Med. Sci.*, cxliii, p. 77, 1912.

²² A preliminary report of these experiments was made at the 1911 Meeting of the Society of Biological Chemists, cf. Proceedings: this *Journal*, xi, p. xxi, 1912.

²³ Benedict: this *Journal*, viii, p. 405, 1910.

²⁴ Folin: *Amer. Journ. of Physiol.*, xiii, p. 45, 1905.

²⁵ Benedict and Myers: *ibid.*, xviii, p. 397, 1907.

²⁶ Harvey: *Arch. of Int. Med.*, vi, p. 12, 1910.

²⁷ Drushel: *Amer. Journ. of Sci.*, xxvi, p. 555, 1908; also Myers: this *Journal*, vi, p. 122, 1909.

ously introducing an important factor into the figures for the nitrogen elimination. However, starving animals would have been less satisfactory, for several reasons, the most important of which is the elimination of creatine which develops very quickly in the rabbit during inanition.

The 350–400 grams of carrots (contain 0.6–0.7 gram N) would appear to furnish a fairly adequate dietary for the rabbit, generally close to 100 calories per kilogram, with 10 per cent in the form of protein. The rabbit with its large skin area should require a comparatively high caloric intake, but this would appear to have been well supplied. Owing to the exhaustion of the old carrot supply (July 1911), rabbits O, P and Q were fed 300 grams of new roots and 200 grams of the green “tops,” thus making the 500 grams recorded in the tables. No detailed protocols, aside from the tabulated results of the urine analyses, appear to be necessary.

The first series of experiments was carried out at the Bender Laboratory at the suggestion of Dr. Ordway with the idea that some correlation might be observed between our chemical findings and the histological data obtained in independent experiments on the same animals by Ordway, Kellert and Huested.²⁵ We were able to detect little relationship between the lesions observed in the various organs and the composition of the urine. Our chemical data were found to be chiefly of interest from the standpoint of creatinine, and on this account figures for the potassium, chlorides, phosphates and likewise ammonia have not been included.

Below is given, however, a brief summary of the results obtained for the elimination of these constituents. Data on the excretion of potassium were obtained in the first seven experiments. The excretion appeared to follow closely in all cases the food intake, although this was rather high to have allowed the demonstration of changes due to fever or accompanying conditions. In general, with the development of fever, there was an increase in the chloride elimination, followed by a very decided decrease not accounted for by the decreased intake. This is believed to have been due to a decreased kidney (tubular) permeability. The phosphate elimination showed an increase during the fever period in certain of the experiments, while in others there was little change.

The figures for ammonia are also not recorded, for the reason that with one possible exception, Rabbit M, no indication was observed of an increased excretion of ammonia. Normally, the rabbit eliminates practically no ammonia, as might be expected from the nature of its dietary. Though the reaction of the urine was frequently observed to be acid at the height of the fever, sufficient time hardly elapsed to have caused a very great depletion of the supply of basic elements in the body.

²⁵ Ordway, Kellert and Huested: *Journ. of Med. Res.*, xxviii, p. 41, 1913.

TABLE I. *Rabbit A.*

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	CREAT- ININE N	CREA- TINE N
			p.m.	a.m.				
February and March	kgms.	grams	°C.	°C.	cc.		mgms.	mgms.
20	1.40	350	38.3	36.7	235	1.018	18.4	0
21	1.39	350	39.5	37.8	250		12.3	0
22	1.43	350	37.8	36.1	275	1.012	19.0	0
23	1.36	350	37.2	38.1	250	1.014	16.1	0
24	1.40	350	38.7	38.6	240	1.016	15.3	0
Average 15-24.....					247	1.015	17.2	
25	1.40	350	37.8	37.8	240	1.021	22.6	0
26	1.36	350	38.5	38.3	260	1.020	19.6	0
27	1.36	350		40.1	250		18.1	0
28	1.34	200		40.1	200	1.014	21.5	0
1	1.24	180	40.6	40.3	75	1.024	22.2	0
2	1.28	70	40.1	40.0	60	1.028	20.7	0
3	1.29	160	39.8	38.6	120	1.020	18.4	0
4	1.32	280	39.2	38.6	175	1.025	16.7	2.1
5	1.26	300	40.8	38.7	235	1.014	18.2	2.1
6	1.24	300	38.3	40.0	200	1.020	16.4	0
7	1.25	300	39.5	39.3	200	1.016	17.4	0.5

TABLE II. *Rabbit C.*

February and March	kgms.	grams	°C.	°C.	cc.		mgms.	mgms.
20	1.75	350	37.5		218	1.017	25.8	
21	1.76	350	37.5	37.8	275	1.019	25.8	
22	1.72	350	37.8	37.8	275	1.019	25.8	
23	1.77	350	38.3	38.8	260	1.020	18.4	
24	1.75	350	39.1	38.4	220	1.022	28.2	
Average 15-24.....					246	1.018	25.4	
25	1.65	350	38.6		300	1.016	32.6	0
26	1.66	350	38.5		250	1.020	25.4	0
27	1.67	350	38.2		350		33.2	0
28	1.70	350	39.6		275	1.018	22.3	0
1	1.65	300	40.6	38.6	235	1.012	37.5	0
2	1.52	280	39.9	40.5	50	1.030	21.8	0
3	1.47	90	41.0	40.3	60	1.035	39.4	0
4	1.47	80	40.6	38.3	50	1.036	29.1	5.6

Rabbit A inoculated subcutaneously on February 24 with 0.5 cc. of a 24-hour bouillon culture of *Bacillus suipesticus*. Pulse on February 21, 180; on February 28, 280. Animal recovered.

Rabbit C inoculated subcutaneously on February 24 with 0.5 cc. of a 24-hour bouillon culture of *Bacillus suipesticus*.

TABLE III.
Rabbit II.

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SPEC. GR.	TOTAL N	UREA N	CREAT- ININE N	CREAT- ININE N	CREATINE N
			p.m.	a.m.			grams	grams	per cent	mgms.	mgms.
June	kgms.	grams	*C.	*C.	cc.						
1	1.70	350	39.8	39.5	230	1.018	0.70	0.45	60	25.1	3.6
2	1.71	350	39.5	39.2	250	1.013	0.55	0.33	60	25.1	4.6
3	1.69	350	40.2	40.1	230	1.010	0.50	0.30	60	25.8	5.1
4	1.70	350	39.7	39.7	230	1.015	0.42	0.32	76	25.8	6.0
Aver.											
1-4					250	1.013	0.53	0.35	66	25.5	5.0
5	1.67	343	40.9	40.3	230	1.015	0.63	0.48	76	31.7	5.0
6	1.68	347	41.0	41.4	250	1.018	0.72	0.61	84	31.7	4.4
7	1.64	200	42.0	41.8	152	1.017	0.69	0.62	90	34.7	5.0
8	1.52	0	41.7	41.1	70	1.015	0.83	0.70	84	35.4	4.2
9	1.47	H ₂ O, 16 cc.	40.0	39.1	33	1.015	0.25	0.24	96	17.2	6.9
											5.4

Rabbit II inoculated subcutaneously on June 4 at 12.30 p.m. with 0.5 cc. of 24-hour bouillon culture of *Bacillus suispesticus*. Urine strongly acid on last two days, and on last day contained albumin, a few pus cells and many hyaline casts. Rabbit died at 10 a.m. on June 9.

TABLE IV.
Rabbit I.

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	TOTAL N	UREA		CREAT- ININE N	CREAT- ININE N	
			p.m.	a.m.				grams	per cent		mgms.	per cent
June		grams	°C.	°C.	cc.							
1	2.02	350	39.2	38.9	230	1.016	0.92	0.64	70	27.8	3.0	0
2	1.99	350	39.2	39.0	275	1.017	0.78	0.66	84	27.8	3.5	0
3	2.00	350	39.5	39.2	230	1.017	0.91	0.70	77	25.8	2.8	0
4	1.99	350	39.1	39.1	265	1.017	0.79	0.68	86	26.1	3.3	0
Aver. 1-4					250	1.017	0.85	0.67	80	26.9	3.1	
5	1.96	350	39.8	39.9	237	1.017	0.94	0.73	77	30.1	3.2	4.0
6	2.05	350	39.5	40.0	185	1.020	0.56	0.42	75	25.8	4.6	3.3
7	2.05	350	39.8	40.1	265	1.015	0.70	0.57	80	29.1	4.1	0
8	1.83	0	41.7	40.7	300	1.013	1.04	0.77	74	34.7	3.3	0
9	1.70	H ₂ O, 24 cc.	40.3	37.6	96	1.012	0.88	0.63	71	30.1	3.4	0

Rabbit I inoculated subcutaneously on June 4 at 12.30 p.m. with 0.5 cc. of a 24-hour bouillon culture of *Bacillus suispesticus*. Urine faintly acid on last day, and on the last two days contained albumin. Animal died at 2 p.m. on June 9 with convulsions; was found to be pregnant.

TABLE V.
Rabbit K.

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	TOTAL N	UREA		CREAT- ININE N	CREAT- ININE N	
			p.m.	a.m.				grams	per cent.		mgms.	per cent.
June and July	kgms.	grams	°C.	°C.	cc.		grams	grams	per cent.	mgms.	mgms.	per cent.
	1.84	400	40.2	40.0	315	1.017	1.05	0.85	82	28.8	2.7	10.3
	1.84	400	40.0	39.9	315	1.015	0.75	0.70	93	32.4	4.3	2.1
	1.84	400	40.0	40.0	320	1.015	0.94	0.85	90	32.4	3.4	7.4
	1.85	400	40.0	40.0	282	1.017	0.84	0.74	90	30.5	3.6	10.0
	1.84	400	39.8	39.8	310	1.015	0.85	0.73	86	28.9	3.4	6.8
Average 22-26.....					308	1.016	0.89	0.78	87	30.6	3.4	6.9
27	1.84	400	40.2	40.2	300	1.015	0.94	0.78	83	31.9	3.3	0
28	1.86	400	41.0	39.8	285	1.015	1.03	0.89	86	30.1	3.0	0
29	1.85	400	40.0	40.8	295	1.014	0.86	0.75	89	30.5	3.5	1.9
30	1.84	400	40.7	41.4	275	1.015	0.87	0.74	85	30.8	3.5	2.6
1	1.66	150 H ₂ O, 25 cc.	42.7	39.9	158	1.019	1.33	1.09	80	38.3	3.0	4.6

Rabbit K inoculated in the usual way at 5.30 p.m. on June 26; pregnant and aborted on July 1; died at 9.45 a.m. on July 2. The fact that creatine was excreted during the control period finds probable explanation in the fact that the animal was pregnant. This might also be a possible explanation of the excretion of creatine by the previous animal after inoculation, but previous to the onset of the fever.³¹

³¹ Cf. Mollanby: *Proc. Roy. Soc., B*, lxxxvi, p. 88, 1913.

TABLE VI.
Rabbit M.

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	TOTAL N	UREA N	UREA N	CREAT- ININE N	CREAT- ININE N	CREATINE N
			P.M.	A.M.								
	kgms.	grams	°C.	°C.	cc.		grams	grams	per cent	mgms.	per cent	mgms.
June and July												
22	1.85	400	39.7	39.6	315	1.015	0.51	0.49	96	25.4	5.0	0
23	1.82	400	39.7	39.7	280	1.015	0.55	0.46	83	24.2	4.4	0
24	1.85	400	40.0	39.6	298	1.015	0.52	0.44	84	24.0	4.6	0
25	1.84	400	39.5		295	1.015	0.57	0.45	80	24.5	4.3	0
26	1.82	400	39.6	39.5	320	1.015	0.53	0.45	85	24.8	4.7	0
Average 22-26.					302	1.015	0.53	0.46	87	24.6	4.6	0
27	1.82	400	40.0	40.1	307	1.017	0.50	0.44	88	26.7	5.3	0
28	1.80	390	40.0	40.0	253	1.014	0.64	0.52	81	26.3	4.1	0
29	1.84	380	40.9	41.5	307	1.014	0.60	0.49	81	29.7	4.9	0
30	1.79	0	42.0	40.8	167	1.013	0.91	0.79	87	32.4	3.5	0
1	1.61	0	39.5		25	acid		0.34		5.6		2

Rabbit M inoculated in the usual way at 5.30 p.m. on June 26; small amount of albumin detected in the urine on June 29; urine on morning of last two days observed to be acid; rabbit died in convulsions at 3 p.m. on June 30.

TABLE VII.
Rabbit O.

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	TOTAL N		UREA N		CREAT- ININE N		CREATINE N
			p.m.	a.m.			grams	per cent	grams	per cent	mgms.	per cent	
July					cc.								
16	1.95	500	40.0	39.5	365	1.019	0.87	0.66	0.66	76	28.8	3.1	0
17	1.94	500	40.0	39.8	335	1.024	1.13	0.88	0.88	78	31.0	2.7	0
18	1.95	500	39.8	39.3	300	1.021	0.83	0.62	0.62	74	31.0	3.7	0
19	1.95	500	39.6	39.5	367	1.018	0.86	0.69	0.69	80	31.3	3.7	0
20	1.95	500	39.9	39.6	330	1.022	0.97	0.68	0.68	70	30.1	3.1	0
Aver. 9-20.					328	1.021	0.85	0.64	0.64	76	28.2	3.4	0
21	1.95	500	40.0	40.3	330	1.021	1.05	0.82	0.82	78	28.9	2.7	0
22	1.95	500	39.7	39.7	306	1.022	0.96	0.74	0.74	77	30.1	3.0	0
23	1.97	500	40.8	40.7	208	1.021	0.90	0.68	0.68	76	31.0	3.4	0
24	1.95	500	41.4	41.1	320	1.019	0.96	0.66	0.66	69	36.3	3.8	0
25	1.92	refused	42.2	41.1	140	1.019	0.94	0.79	0.79	73	38.3	4.0	0
26	1.81	food but	41.5	40.9	190	1.014	1.65	1.49	1.49	90	47.9	2.9	0
27	1.75	drank H ₂ O	41.1	40.7	50	1.028	1.28	1.10	1.10	90	33.4	2.6	0

Rabbit O received 5 cc. of a bouillon culture of *Bacillus suispesticus* by mouth at 5 p.m. July 12 on an empty stomach but no infection apparently ensued. At 5.30 p.m. on July 20, 0.2 cc. of a 24-hour bouillon culture was given subcutaneously. Animal died at 12.30 p.m. July 27.

TABLE VIII.

Rabbit P.

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	TOTAL N	UREA N	UREA N	CREAT- ININE N	CREAT- ININE N	CREATINE N
			p.m.	a.m.								
July	kgms.	grams	°C.	°C.	cc.		grams	grams	per cent	mgms.	per cent	
9	2.56	500	39.5	39.5	305	1.021	0.68	0.57	88	35.1	5.2	0
10	2.34	500	40.0	40.0	235	1.026	0.86	0.59	69	35.1	4.1	0
11	2.37	500	41.1	39.2	305	1.020	0.75	0.61	81	35.1	4.7	0
12	2.38	500	40.0	39.6	265	1.025	0.92	0.68	74	35.1	3.8	0
Average 9-12 ...					282	1.023	0.80	0.61	76	35.1	4.4	0
13	2.36	500	40.2	40.3	300	1.023	0.85	0.65	77	35.1	4.1	0
14	2.40	500	39.7	39.2	325	1.024	0.83	0.61	74	35.1	4.2	0
15	2.36	500	39.7	40.6	306	1.019	0.59	0.44	75	27.0	4.6	0
16	2.32	300	41.9	41.9	325	1.019	1.05	0.81	77	39.7	3.8	0
17	2.22	100	41.9	41.7	107	1.020	1.07	0.82	77	40.5	3.8	0
18	2.17	0	41.3		29		0.26	0.20	77	16.7	6.4	0

Rabbit P received 0.2 cc. of the usual culture subcutaneously at 5 p.m. July 12 and died during the night of July 17.

TABLE IX.

Rabbit Q.

DATE 1911	BODY WEIGHT kgs.	CARROT DIET grams	TEMPERATURE		VOL. OF URINE cc.	SP. GR.	TOTAL N		UREA N		CREAT- ININE N	
			p.m.	a.m.			grams	per cent	grams	per cent	mgms.	mgms.
July			°C.	°C.								
17	1.63	500	40.3	39.8	285	1.030	0.76	0.51	67			
18	1.60	500	40.0	39.5	343	1.018	0.46	0.32	70			
19	1.59	500	40.0	40.0	342	1.019	0.65	0.50	77			
20	1.59	500	40.7	40.0	330	1.020	0.54	0.41	76			
Average 9-20...												
					303	1.020	0.56	0.38	68	days 8-14		
21	1.57	500	40.0	40.2	325	1.021	0.61	0.44	72	17.6	2.9	0
22	1.61	500	40.1	40.1	226	1.025	0.59	0.45	76	17.6	3.0	0
23	1.62	500	40.0	40.0	296	1.024	0.84	0.64	76	20.2	2.4	0
24	1.57	500	41.2	40.9	320	1.019	0.62	0.48	77	21.3	3.4	0
25	1.61	300	40.7	41.7	285	1.020	0.71	0.53	75	21.7	3.1	0
26	1.53	300	42.1	41.4	172	1.022	0.91	0.77	85	26.4	2.9	0
27	1.54	215	41.4	41.1	75	1.027	0.89	0.74	83	25.1	2.8	0
28	1.56	225	41.6	40.6	100	1.040	0.92	0.74	80	25.1	2.7	3.4
29	1.50	70	41.1	38.6	85	1.026	1.50	1.25	83	26.3	11.8	15.8

Rabbit Q received 5 cc. of the usual bouillon culture by mouth at 5 p.m. July 12 on an empty stomach, but no infection followed. On July 21 the animal was given a subcutaneous injection of 0.2 cc. of the culture. Rabbit died July 29.

TABLE X.
*Rabbit 45.*³⁰

DATE 1912	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	TOTAL N	CREAT- ININE N	CREAT- ININE N
			p.m.	a.m.					
July and August	kgms.	grams	°C.	°C.	cc.		grams	mgms.	per cent
31	1.87	300	39.8	39.0	236	1.017	0.67	22.0	3.3
1	1.74	300	39.5	39.3	268	1.017	0.84	27.0	3.2
2	1.57	350	39.6	39.1	230	1.018	0.68	23.0	3.4
3	1.70	350	39.9	39.0	242	1.018	0.64	24.7	3.9
4	1.65	350	39.3	39.5	300	1.016	0.68	27.4	3.7
5	1.62	350	39.6	39.5	225	1.017	0.74	27.4	4.0
Average 31-5...					250	1.017	0.71	24.6	3.5
6	1.64	250	40.9	41.1	85	1.023	0.41	23.8	5.8
7	1.56	120	41.0	41.7	40	1.027	0.82	30.6	3.7
8	1.41	110	41.7	43.6	30	1.030	0.99	32.1	3.2
9	1.42	240	39.0	39.1	73	1.031	1.07	28.7	2.7
10	1.52	350	39.2	39.5	183	1.018	0.72	21.2	2.9
11	1.58	350	39.3	39.6	305	1.016	0.70	20.8	3.0

Rabbit 45 was placed in the incubator on the morning of August 5 and kept there continuously for three days, except for about one hour each morning. During the incubator period the animal drank a small amount of water. The first day after being removed from the incubator the rabbit appeared sick, but was apparently normal on the following day.

³⁰ In this last series of experiments we were assisted by Mr. Adolph Bernhard.

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TABLE XI. *Rabbit 47.*

DATE 1913	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	TOTAL N	CREAT- ININE N	CREAT- ININE N
			p.m.	a.m.					
January	<i>kgs.</i>	<i>grams</i>	<i>°C.</i>	<i>°C.</i>	<i>cc.</i>		<i>grams</i>	<i>mgms.</i>	<i>per cent</i>
16	1.58	320	39.4	39.0	225	1.012	0.27	20.3	7.5
17	1.59	340	39.6	39.0	235	1.012	0.33	19.6	5.9
18	1.59	325	39.5	39.0	270	1.010	0.31	16.0	6.5
19	1.56	350	39.7	38.7	268	1.013	0.44	18.4	4.2
20	1.60	350	38.7	39.0	200	1.010	0.33	19.2	5.8
Average 16-20.					240	1.011	0.34	18.7	5.5
21	1.48	110	41.2	40.6	58	1.018	0.33	23.2	7.0
22	1.35	35	40.2	41.7	28	1.028	0.62	25.9	4.2
23	1.33	0	41.6	41.4	25	1.028	0.73	26.9	3.7
24	1.46	200	39.4	38.0	40	1.030	0.82	21.5	2.6
25	1.56	310	38.0	39.1	140	1.020	0.44	20.1	4.6

Rabbit 47 was placed in the incubator at 2 p.m. on January 20 and kept there continuously, except for the time necessary to take temperatures, etc., until 9 a.m. on the 23rd. On the last two of these three days the reaction of the urine was observed to be acid.

TABLE XII. *Rabbit 49.*

DATE 1913	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP. GR.	TOTAL N	CREAT- ININE N	CREAT- ININE N
			p.m.	a.m.					
January	<i>kgs.</i>	<i>grams</i>	<i>°C.</i>	<i>°C.</i>	<i>cc.</i>		<i>grams</i>	<i>mgms.</i>	<i>per cent</i>
16	1.36	350	39.7	39.2	290	1.010	0.12	18.4	15.3
17	1.32	350	39.7	39.3	315	1.011	0.19	21.1	11.1
18	1.32	350	40.0	39.3	230	1.012	0.23	18.3	8.0
19	1.34	350	39.7	39.1	245	1.012	0.28	20.0	7.1
20	1.42	350	38.1	39.5	180	1.016	0.22	16.4	7.5
Average 16-20.					252	1.012	0.21	18.8	9.0
21	1.39	350	42.0	39.1	220	1.010	0.35	21.2	6.1
22	1.26	80	40.7	42.1	30	1.022	0.22	18.8	8.5
23	1.29	50	41.5	41.5	35	1.032	0.72	24.1	3.3
24	1.36	350	38.9	39.0	205	1.016	0.60	21.4	3.6
25	1.45	350	39.1	39.1	100	1.027	0.19	15.0	7.9

Rabbit 49 was placed in the incubator at 2 p.m. on January 23. The temperature at 8.45 p.m. was found to be 42.1°C., and it was thought best to remove the animal to the room. The next morning (22nd) the rabbit was again placed in the incubator and kept there for the two following days.

Discussion.

The results of the foregoing experiments can most advantageously be discussed together. The parallel between the body temperature and the total nitrogen, urea, and creatinine is quite striking, though in considerable measure probably due to the shortness of the fever, generally five to eight days. In all cases the highest elimination of nitrogen has been found to coincide with the highest temperature. The relation between the total nitrogen and urea, as shown by the percentage of urea nitrogen, does not markedly differ from the normal. In general, during the fever period there was a slight increase in the per cent of urea nitrogen. At the height of the fever this was sometimes accompanied by a still further increase, and in other cases by a decrease.

The relation between the body temperature and the amount of the creatinine elimination is most interesting. As soon as the body temperature rises noticeably above the normal level, there is an accompanying increase in the excretion of creatinine, and with a decline in the febrile temperature there is an immediate fall. Though, in general, the creatinine follows the increased total nitrogen excretion, there is a very noticeable lag at the height of the fever, the average figures disclosing a fall of from 3.8 per cent to 3.3 per cent in terms of total nitrogen. The maximum temperature, about 42°C., was found to be accompanied by the highest creatinine elimination, the percentage increase over the control elimination averaging 36 per cent—64 per cent in one case—as recorded in Table XIII.

Creatine was generally found to be eliminated after the crisis of the disease. This was also the case in one other experiment not reported, Rabbit L. The recent experiments of Myers and Fine¹¹ on starving rabbits make it evident that the creatine of the urine, at least in that condition, has the origin generally assumed, viz., from the setting free of creatine in the absorption of muscle tissue. Though creatine may be an index as to the amount of a certain kind of abnormal catabolism, the creatine itself bears no uniform relation to the endogenous total nitrogen excretion.

To secure further light on the factors causing the increased elimination of creatinine during fever, three animals were placed in a

¹¹ Myers and Fine: *Proc. Soc. Exp. Biol. and Med.*, x, p. 12, 1912.

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large incubator at about 38.5°C. As is well known, the rabbit is easily susceptible to external changes in temperature and in this way a typical hyperthermia was easily produced with maximum temperatures varying between 41.7° and 43.3°C.

As is shown in Tables X, XI and XII, the rise in temperature here was likewise found to be accompanied by an increased elimination of creatinine, the maximum output being noted on the day of the highest temperature. The increased excretion was of the same intensity as that observed in the previous series of experiments. It is of interest to note that here no creatine was found to appear in the urine, possibly indicating that there was no abnormal destruction of muscle tissue. Inasmuch as no creatine was found no tabulation was made. The maximum total nitrogen excretion in this series did not appear at the time of the highest temperature, but on the day following. The coincidence in the previous series finds probable explanation in the fact that the highest temperature and the highest total nitrogen just preceded the premortal fall.

TABLE XIII.

EXPERIMENT	AVERAGE DAILY TOTAL N CONTROL PERIOD	HIGHEST TOTAL N FEVER PERIOD	AVERAGE DAILY CREATININE N CONTROL PERIOD	HIGHEST CREATININE N FEVER PERIOD	INCREASED CREATININE EXCRETION DUE TO FEVER
	grams	grams	mgms.	mgms.	per cent
A			17.2	22.2	29
C			25.4	39.4	55
H	0.53	0.83	25.5	35.4	39
I	0.85	1.04	26.9	34.7	29
K	0.89	1.33	30.6	38.3	25
M	0.53	0.91	24.6	32.4	32
O	0.85	1.65	29.2	47.9	64
P	0.80	1.07	35.1	40.5	15
Q	0.56	1.50	19.5	26.4	35
Average increased excretion of creatinine					36
45	0.71	1.07	24.6	32.1	31
47	0.34	0.82	18.7	26.9	44
49	0.21	0.72	18.8	24.1	28
Average increased excretion of creatinine					34

In the above table are summarized the more important data in connection with the excretion of creatinine. Percentage figures for the increased excretion of total nitrogen during the height of the fever are not given owing to the fact that it appeared impossible to compute reliable figures. During the control period a number of the animals showed a plus nitrogen balance, with nitrogen intakes varying between 0.6 and 0.7 gram. Furthermore, during the fever period, they were burning increased amounts of their own tissue due in part to the decreased food intake. However, it appears that there was a greater increase in the excretion of nitrogen during pyrexia in the first series of experiments than in the second.

CONCLUSIONS.

The excretion of creatinine closely follows the rise in temperature during fever, whether the hyperthermia is of infective origin or artificially induced. The highest continued temperature (about 42°C.) has been found to be accompanied by the highest creatinine elimination.

In nine experiments on rabbits inoculated with *Bacillus suispesticus*, the percentage increase at the height of the fever over the control elimination averaged 36 per cent. In three experiments, where hyperthermia was artificially induced, the average increase was 34 per cent. This is believed to show that the increased elimination of creatinine is due entirely to the hyperthermia.

The view is expressed that the increased creatinine elimination during fever still represents the normal endogenous metabolism, which is proceeding here at an abnormal intensity due to the high temperature. It is possible that this is in accord with the law of the increased velocity of chemical reactions at increased temperatures.

It is suggested that the amount of the increased creatinine excretion may be of value in indicating the increase in nitrogenous metabolism due to simple pyrexia. The increased excretion of nitrogen in physiological fever corresponds very well with the increased excretion of creatinine (35 per cent), but in toxic fevers the excretion of nitrogen may be much greater.

In the series of toxic fevers, creatine was generally found to be excreted, and when present was observed, as a rule, following the

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crisis of the fever. In the series of physiological fevers an elimination of creatine was not detected. It is reasonable to believe that creatine is excreted during fever, because the protein is drawn upon to supply unusual demands (*e.g.*, to supply energy in the absence of carbohydrate), and in this abnormal catabolism, creatine is set free more rapidly than the body can oxidize it.

In fever, creatinine, though increased, still appears to indicate the amount of a certain type of normal endogenous metabolism, while creatine possibly indicates the amount of abnormal endogenous metabolism.

THE NORMAL PROTEIN METABOLISM OF THE RAT.

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The highly interesting results obtained by Mendel and Osborne¹ on feeding single specific proteins to rats lend added interest to any detailed information that may be obtained concerning the nature of the metabolism of the rat as compared with that of any other animal or of man. The small size of the animal has heretofore prevented the accumulation of much information of a quantitative nature. The total urinary nitrogen determinations of Mendel and Osborne and those of Hatai² represent so far as we know all that has been done in this line. By means of the new microchemical method,³ recently published from this laboratory it is possible to obtain more detailed figures. Because of the limited quantity of urine comprising the twenty-four-hour amounts, some changes in these methods were found necessary. In every case the twenty-four-hour urine of each animal (5-50 cc. in amount) was made up to 50 cc. From this stock solution portions were taken directly for ammonia, uric acid, creatinine and creatine determinations. For total nitrogen and urea determinations a dilution of a part of this solution to twice its volume brought the nitrogen content to the desired amount ($1 \pm$ mgm. per 1 cc.). In the smallest rats this second dilution was unnecessary, the quantity being sufficiently reduced in the first dilution.

The uric acid analysis followed the general scheme of its determinations in human urine as published by Folin and Denis.⁴ Ten cubic centimeters of rat urine were found to contain a sufficient quantity of uric acid for the determination. A drop of acetic acid

¹ Carnegie Institution of Washington, publication 156, Pts. I and II.

² *Amer. Journ. of Physiol.*, xiv, p. 102, 1905.

³ *This Journal*, xi, 1912.

⁴ *Ibid.*, xiv, p. 95, 1913.

(instead of hydrochloric) was used to acidify when driving off the hydrogen sulphide. After the reaction with the uric acid reagent and sodium carbonate the blue solution was filtered into a 25 cc. volumetric flask and made up to the mark with washings. It was then read against a standard of 1 mgm. of uric acid in 100 cc.

The creatinine determinations offered at the beginning considerable difficulty owing to the small amount of creatinine and its great dilution in rat urine. The original standard, potassium bichromate solution, could not be used and had to be replaced by creatinine solutions of known concentration. Other factors had to be modified—the amounts of picric acid and alkali, and the time necessary for the development of the maximum color had to be worked out. Our standard creatinine solutions were made of approximately the same concentration as the urine. These solutions and the urine were then treated exactly alike and, by developing the color simultaneously in both, constant and seemingly reliable results were obtained. The modified procedure is as follows:

To 5 cc. of urine and to 5 cc. of the standard creatinine solution, each in a 25 cc. volumetric flask, add 2.5 cc. of saturated picric acid solution from a burette, and then 1 cc. of 10 per cent sodium hydroxide solution. Let stand for ten minutes, fill up to the mark with water and then determine the color as in the original method.

The creatine determination was made, in terms of creatinine, by the same method and appeared as an addition to the previously obtained creatinine figure. It was converted into creatinine by adding 0.5 cc. of 2*N* HCl to 5 cc. of urine in a 100 cc. Erlenmeyer flask, evaporating to rather less than 2 cc. volume on a water bath, and continued heating at this volume for two hours. To maintain the volume a small top-shaped glass bulb was inserted in the mouth of the flask for condensation. At the end of the time indicated the volume was again made up to approximately 5 cc. by adding water to the flask on a balance until the weight was the sum of 5 grams and the previously-taken weight of the flask. Beyond this the treatment was exactly the same as with creatinine except that an additional 0.5 cc. of NaOH was added to neutralize the HCl used.

Each rat was kept in a specially constructed small cage resting on top of a funnel. Each day's urine was collected in 2 cc. of normal hydrochloric acid. The twenty-four-hour aspect is only approximate since no attempt was made to have the bladder emptied at the end of each day. The duration of the experiment is long enough, however, so that the average of all the figures should come close to the true twenty-four-hour results. The urines were analyzed in the case of rats M, X and A over a period of nine days, and in the case of rat G, fifteen days.

In tables 1 and 2 are given the analyses of a series of approximately twenty-four-hour urines obtained from two large mature rats, a female (M) weighing 290 grams and a male (X) weighing 197 grams. To save space only the last six days are recorded.

Table 3 represents a summary of the results recorded in tables 1 and 2, together with summaries of the results obtained from two young growing rats, A and G, weighing 40 to 50 grams. We have made several other series of similar studies but these deal chiefly with tumor-bearing rats and will be described elsewhere by Ordway and Morris.

The rats were kept on a purine-free diet consisting of powdered crackers and water.

It will be seen, from examination of the average results, that the percentage composition of rat urine differs but little from that of human urine. Being small animals but voracious feeders the total nitrogen per kilo of body weight is much larger than in the case of man. The percentage relationship between the amounts of total nitrogen, urea nitrogen and ammonia nitrogen is almost the same as in man. Creatinine nitrogen shows a somewhat larger value for the rats, reaching 15 mgm. per kilo in the mature rats, while man usually eliminates 7-11 mgm. Small amounts of creatine were always found in the urine. This is rather interesting in view of the excessive feeding of rats and the suggestion of Folin and Denis⁵ that the creatine in the urine of children might be due to an "excessively high level of protein consumption."

The most striking and interesting feature of the analyses is the fact that the urine of rats contains quite as much uric acid in proportion to body weight as does human urine. Through the

⁵ This *Journal*, xi, p. 253, 1912.

TABLE 1.
Female rat weighing 290 grams (M).

DATE (DEC.)	16		18		19		20		21		AV. 24 HOURS	
	mgm.	per cent	mgm.	per cent	mgm.	per cent	mgm.	per cent	mgm.	per cent	mgm.	per cent
Total N.....	172.5	100.0	184.5	100.0	165.0	100.0	190.5	100.0	155.0	100.0	173.5	100.0
Urea N.....	147.9	86.0	139.1	75.4	130.0	78.8	150.0	78.7	103.8	67.0	143.2	77.3
Ammonia N.....	9.1	5.3	9.4	5.1	9.5	5.8	9.4	4.9	8.2	5.3	9.1	5.2
Uric Acid N.....	0.52	0.3	0.73	0.4	0.71	0.43	0.83	0.43	0.65	0.42	0.69	0.40
Creatinine N.....	4.8	2.8	4.7	2.6	3.9	2.4	4.3	2.2	4.7	3.0	4.5	2.65
Creatinine + Creatine N.....	5.0	2.9	4.6	2.6	3.6	2.3	4.7	2.5	4.7	3.0	4.7	2.71

TABLE 2.
Male rat weighing 197 grams (X).

DATE (DEC.)	16		17		18		19		20		21		AV. 24 HOURS	
	mgm.	per cent	mgm.	per cent	mgm.	per cent	mgm.	per cent	mgm.	per cent	mgm.	per cent	mgm.	per cent
Total N.....	144.0	100.0	127.0	100.0	119.0	100.0	118.0	100.0	116.0	100.0	132.0	100.0	126.0	100.0
Urea N.....	115.3	80.1	109.8	86.5	102.7	86.3	88.7	75.2	101.0	87.1	118.1	89.5	105.9	84.0
Ammonia N.....	8.2	5.7	7.1	5.6	6.6	5.5	6.3	5.4	5.4	4.7	6.9	5.2	6.7	5.3
Uric Acid N.....	0.43	0.3	0.75	0.59	0.62	0.52	0.43	0.36	0.62	0.54	0.52	0.41	0.52	0.41
Creatinine N.....	3.1	2.1	3.3	2.6	2.6	2.2	3.0	2.5	2.4	2.1	3.2	2.4	2.9	2.30
Creatinine + Creatine N.....	3.2	2.2	3.7	2.9	2.9	2.5	2.7	2.2	2.4	2.1	3.3	2.5	3.0	2.38

TABLE 3.
 Summary of percentages.

	BAT										
	M			X			A			G	
	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Av.
Weight.....	292.0	289.0	290.5	197.0	197.0	197.0	42.7	41.0	41.9	51.5-	49.0
Urea N.....	86.0	67.0	77.3	89.5	75.2	84.0	78.2	64.1	71.3	87.7	76.3
Ammonia N.....	8.6	4.9	5.2	8.5	5.2	5.3	11.7	6.1	9.0	10.5	7.0
Uric Acid N.....	0.43	0.29	0.40	0.59	0.30	0.41	0.47	0.21	0.35	0.67	0.48
Creatinine N.....	3.0	2.2	2.65	2.6	2.1	2.30	1.3	1.0	1.08	1.7	1.37
Creatinine N + Creatine N.....	3.1	2.3	2.71	2.9	2.1	2.38	1.5	1.1	1.16	1.8	1.44

Summary in grams per kilo body weight.*

	BAT					
	M	X	A	G		
Total N.....	0.598	0.639	1.068	0.594		
Urea N.....	0.463	0.537	0.761	0.453		
Ammonia N.....	0.031	0.034	0.096	0.041		
Uric Acid N.....	0.0024	0.0026	0.0037	0.0029		
Creatinine N.....	0.0155	0.0147	0.0115	0.0082		
Creatinine N + Creatine N.....	0.0162	0.0148	0.0124	0.0086		

* (Figures based on average weights, both of animals and metabolism products.)

investigations of Wiechowski⁶ and more recently of Hunter and Givens⁷ we have learned that mammals other than man convert the greater part of the uric acid into allantoin, and the urines of such animals therefore contain very little if any uric acid. The metabolism of rats is, however, in this respect like that of man.

In view of this highly curious similarity it seemed necessary also to determine whether the blood of rats is as rich in uric acid as is human blood. Folin and Denis⁸ have recently shown that domestic animals, whose urine contains allantoin instead of uric acid, uniformly show mere traces of uric acid in the blood. The blood from six full-grown rats was collected over a little powdered potassium oxalate, and the uric acid, total non-protein nitrogen and urea were determined by the methods of Folin and Denis. The figures obtained for 100 grams of blood were as follows: Uric acid, 2 mgm.; non-protein nitrogen, 38; urea, 22. The experiment was repeated twice, each time using for the analysis the mixed blood of six normal white rats, and 2.4 mgm. and 2.5 mgm. respectively of uric acid per 100 grams of blood were found. These are substantially the same figures as Folin and Denis found for normal human blood. The purine metabolism of rats is, therefore, like that of man and unlike that of other mammals hitherto investigated. Jones and Rohd⁹ published some experiments on purine ferments of the rat a few years ago, and one of the conclusions reached is interesting in connection with our results: "The results of this work show that the organ extracts of the rat jointly and severally are incapable of exhibiting either adenase or xanthoxidase. There is, therefore, no way for uric acid to be formed by the purine ferments in extracts of the organs of the animal. Nevertheless rats' urine contains uric acid. From 50 cc. of urine we were able to isolate enough uric acid for complete identification. Presumably the organs also contain uric acid which might be detected by methods of sufficient refinement but the substance cannot be produced by the action of organ extracts on purine bases."

⁶ *Biochem. Zeitschr.*, xxv, p. 433, 1910.

⁷ *This Journal*, xiii, p. 372, 1912.

⁸ *Ibid.*, xiv, p. 31, 1913.

⁹ *Ibid.*, vii, p. 237, 1909.

It seems rather remarkable that the one animal which (excepting man) produces the most uric acid in the course of normal metabolism should lack the ferments capable of producing it. In this connection we can state that investigations conducted by J. B. Sumner in this laboratory have shown that aqueous extracts from rat livers are as capable of destroying uric acid as similar extracts obtained from the livers of cats and sheep. The significance of "purine ferments" as obtained from organ extracts in relation to the formation and elimination of uric acid in the course of normal metabolism is, therefore, far from clear.

IS NARCOSIS DUE TO ASPHYXIATION?

BY JACQUES LOEB AND HARDOLPH WASTENEYS.

(*From the Rockefeller Institute for Medical Research, New York.*)

(Received for publication, April 30, 1913.)

1. The idea that the phenomenon of narcosis might be caused by an interference with oxidation is an old one. Recently it has been advocated by Verworn who tried to support it by indirect evidence.¹ Yet it is obvious that if we wish to ascertain whether or not narcosis is due to a diminution in the rate of oxidations (or, as Verworn expresses it, to an asphyxiation) there is only one way to decide the question, namely by comparing the rate of oxidations in narcotized organisms with that in non-narcotized.

If we wish to carry out such experiments we must keep in mind that organisms which are capable of muscular action cannot well be used for this purpose since we know that muscular activity can easily raise the rate of oxidation in the animal body one hundred per cent. Since the narcotics cause muscular action to cease, a narcotized animal will consume less oxygen than a free-moving animal; but it would be a mistake to state that this diminution in the consumption of oxygen was the cause of narcosis. It is the effect and not the cause of narcosis. If we wish to test the asphyxiation hypothesis of narcosis we must use organisms which possess no muscles and in which therefore this source of error does not interfere with the result. The best material for this purpose is offered in the fertilized eggs. Loeb had shown in 1895 that the eggs of the sea urchin and fish cannot develop if the oxygen is withdrawn² and this result has since been extended to a number of eggs of different forms. The same author showed that the addition of a small amount of KCN also inhibits segmentation. We shall see later that KCN only has this effect if it depresses the rate of oxidations in the egg below a certain level. The suppression of

¹ Verworn: *The Harvey Lectures*, 1911-12, p. 52.

² Loeb: *Pflüger's Archiv*, lxii, p. 249, 1895.

segmentation by lack of oxygen as well as by KCN is a reversible process.

It is also well known that anaesthetics inhibit the segmentation of the fertilized sea urchin egg reversibly. It was, therefore, an easy task to compare the rate of oxidations in equal quantities of fertilized eggs with and without the presence of narcotics. Warburg had already observed that phenyl urethane in the concentration in which it suppressed nuclear and cell division did not diminish the rate of oxidations. Only if an excessive amount of phenyl urethane was added, was a diminution in the rate of oxidations noticeable; but, as he correctly adds, the question is not whether it is at all possible to lower the rate of oxidations by adding narcotics but whether that concentration which suffices for narcosis lowers the rate of oxidations.³

We undertook a series of experiments on the effect of various narcotics upon the rate of oxidations in the newly fertilized eggs of *Strongylocentrotus purpuratus*. Since the method of procedure and the degree of reliability of our method have been described in previous papers no recapitulation of these points is needed.

2. *Experiments with KCN.* We determined the minimum amount of KCN necessary to suppress cell division permanently in the newly fertilized eggs of *S. purpuratus* and found that the addition of 0.7 cc. of 0.01 per cent KCN to 50 cc. of sea water was the minimum amount required. We then determined the influence of various amounts of KCN upon the rate of oxidations. Newly fertilized eggs were divided into equal lots and the amount of oxygen consumed in one hour was determined. Temperature 14°C.

TABLE I.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm.	
1. Normal sea water.....	0.51	1.00
2. 50 cc. sea water + 0.7 cc. 0.01 per cent KCN	0.17	0.33
3. 50 cc. sea water + 0.9 cc. 0.01 per cent KCN	0.15	0.29

In solution 1, the eggs segmented; in solutions 2 and 3 no segmentation occurred. The eggs were transferred from solutions 2 and

³ O. Warburg: *Zeitschr. f. physiol. Chem.*, lxvi, p. 305, 1910.

3 to normal sea water and all developed into normal larvae. The amount of KCN necessary to suppress cell division lowers the rate of oxidations to one-third of the normal amount. A repetition of the experiment confirmed the result.

TABLE II.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm.	
1. Normal sea water.....	0.65	1.00
2. 50 cc. sea water + 0.4 cc. 0.01 per cent KCN	0.34	0.52
3. 50 cc. sea water + 0.7 cc. 0.01 per cent KCN	0.21	0.32
4. 50 cc. sea water + 1.0 cc. 0.01 per cent KCN	0.18	0.28

The addition of 0.4 cc. of KCN only retarded the development but did not suppress it. The addition of 0.7 cc. of KCN suppressed cell division, and the rate of oxidations was again exactly one-third of the normal.

Similar results had previously been obtained by us for the eggs of *Arbacia*.⁴

We can, therefore, state that if the rate of oxidations of the egg is lowered to one-third of the normal amount found at 14°C., the eggs cease to segment. If the prevention of segmentation by narcotics were due to the same influence we should have to expect also a lowering of the rate of oxidations to one-third of the normal rate found at 14°C.

3. We tried a number of narcotics, various alcohols, chloral hydrate, chloroform and ethyl urethane. The minimum amount required to suppress cell division permanently was ascertained for each of these narcotics. The minimal dose varies slightly for the eggs of various individuals.

a. *Chloral hydrate*. The minimum amount required to suppress cell division is 4.2 cc. of 0.5 per cent solution (made up in $\frac{M}{2}$ NaCl + KCl + CaCl₂) to 45.8 cc. of sea water. Temperature 15°C.

⁴ Loeb and Wasteneys: *Biochem. Zeitschr.*, xxxvi, p. 355, 1911.

TABLE III.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm.	
Normal sea water.....	0.65	1.00
2.4 cc. 0.5 per cent chloral hydrate in 50 cc. sea water.....	0.60	0.92
4.2 cc. 0.5 per cent chloral hydrate in 50 cc. sea water.....	0.57	0.88
6.0 cc. 0.5 per cent chloral hydrate in 50 cc. sea water.....	0.57	0.88

In the solution with 4.2 cc. and 6.0 cc. of chloral hydrate the eggs were no longer able to segment; but they segmented and developed promptly when transferred to normal sea water. In the solution with 2.4 cc. of chloral hydrate the eggs segmented. The effect of the chloral hydrate upon oxidations was practically nil. The experiment was repeated.

TABLE IV.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm.	
Normal sea water.....	0.51	1.00
4.2 cc. 0.5 per cent chloral hydrate in 50 cc. sea water.....	0.42	0.89

Although the segmentations were completely suppressed in this solution the influence of the chloral hydrate was practically negligible. We can say with certainty: The narcotic effect of chloral hydrate upon the egg is not due to asphyxiation or a diminution in the rate of oxidations.

b. Ethyl urethane. Three cubic centimeters of a 10 per cent solution (in $\frac{M}{2}$ NaCl + KCl + CaCl₂) in 50 cc. of sea water are sufficient to suppress cell division.

TABLE V.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm.	
Normal sea water.....	0.51	1.00
3 cc. 10 per cent ethyl urethane in 50 cc. sea water	0.46	0.98

With a few exceptions no eggs segmented in ethyl urethane, but all segmented when put back into normal sea water.

c. Chloroform. Seven cubic centimeters of 0.5 per cent CHCl_3 in $\frac{M}{2}$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ were required to suppress cell division.

TABLE VI.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	<i>mgm.</i>	
Normal sea water.....	0.47	1.00
43 cc. sea water + 7 cc. 0.5 per cent chloroform.	0.41	0.87

No segmentation occurred in the eggs in chloroform, but they segmented and developed normally when transferred to normal sea water. The narcotic effect of chloroform is produced without any considerable lowering of the rate of oxidations. The slight lowering observed is not quite but almost within the limits of error and cannot be considered.

d. Propyl alcohol. Minimum amount necessary to suppress cell division is 3.5 cc. of 2 M propyl alcohol in 50 cc. of sea water. In order to avoid lowering of concentration of salts the 2 M solution of alcohol was made up in $\frac{M}{2}$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2$.

TABLE VII.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	<i>mgm.</i>	
In normal sea water.....	1.00	1.00
2.0 cc. 2 M propyl alcohol in 50 cc. sea water....	0.90	0.90
3.5 cc. 2 M propyl alcohol in 50 cc. sea water....	0.92	0.92
5.0 cc. 2 M propyl alcohol in 50 cc. sea water....	0.93	0.93

In 2 cc. of propyl alcohol a few of the eggs (less than 1 per cent) segmented. In 3.5 cc. and 5 cc. of propyl alcohol no egg segmented. A few underwent cytolysis, but as Warburg has shown, and as we were able to confirm, this does not alter the rate of oxidations in the fertilized egg. We therefore see that the narcotic effect of propyl alcohol is not accompanied by any lowering of the rate of oxidations in the egg.

e. Various other alcohols. The narcotic effect of various alcohols follows the rule that each successive alcohol of a series is two or

three times as efficient for narcosis as the previous one. The amount necessary for the suppression of segmentation in the egg of *S. purpuratus* found for the various alcohols was as follows:

	RATE OF EFFICIENCY COMPARED WITH METHYL ALCOHOL.
Methyl alcohol, 4.0 cc. 10 M in 50 cc. sea water.....	1
Ethyl alcohol, 5.0 cc. 4 M in 50 cc. sea water.....	2
Propyl alcohol, 3.5 cc. 2 M in 50 cc. sea water.....	6
Butyl alcohol, 3.5 cc. M in 50 cc. sea water.....	12

TABLE VIII.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm.	
Normal sea water.....	0.87	1.00
4 cc. 10 M methyl alcohol in 50 cc. sea water.....	0.83	0.95
5 cc. 4 M ethyl alcohol in 50 cc. sea water.....	0.73	0.84
7 cc. $\frac{M}{2}$ butyl alcohol in 50 cc. sea water.....	0.66	0.76

No eggs segmented in the solutions containing alcohol except in the methyl alcohol in which 25 per cent of the eggs segmented. When transferred to normal sea water all the eggs segmented. The rate of oxidations in these experiments is too high to account for the narcotic effect on the basis of asphyxiation. The experiment with butyl alcohol was repeated.

TABLE IX.

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm.	
Normal sea water.....	0.84	1.00
6.5 cc. $\frac{M}{2}$ butyl alcohol in 50 cc. sea water.....	0.66	0.79

4. *Theoretical remarks.* The maximal lowering of the rate of oxidations found under the influence of narcotics was 20 per cent; in the majority of cases it was less than this. In the case of propyl alcohol it was less than 10 per cent; in the case of chloral hydrate it was about 10 per cent. Since the temperature coefficient for the rate of oxidations in the eggs is about 2 for 10°C.,⁵ we can pre-

⁵ Loeb and Wasteneys: *Biochem. Zeitschr.*, xxxvi, p. 345, 1911.

duce a lowering of the rate of oxidations of 20 per cent by lowering the temperature two or three degrees, *e.g.*, putting the eggs into sea water of 12° instead of into 15° as in these experiments. The previous experiments of Loeb have shown that the eggs of *S. purpuratus* segment not only at 12° but even at 3°C., when the rate of oxidations is less than one-half of that observed at 15°C. In addition, the experiments with KCN also show that this substance does not suppress cell division until the rate of oxidations is reduced to one-third of the normal value. From these facts we can state with certainty that the effect of narcotics upon the eggs of the sea urchin is not due to asphyxiation; a conclusion which Warburg reached also in his experiments with phenyl urethane.

Kisch⁶ has recently published experiments which show that the photodynamic effect due to oxidations is raised instead of being diminished if narcotics are added to the medium.

SUMMARY.

It is shown that chloral hydrate, ethyl urethane, chloroform and various alcohols produce complete narcosis in the fertilized eggs of the sea urchin without practically lowering the rate of oxidations in the egg.

⁶ Kisch: *Zeitschr. f. Biol.*, ix, p. 399, 1913.

THE CHEMISTRY OF GLUCONEOGENESIS.¹

III. THE FATE OF ISOBUTYRIC, ISOVALERIANIC AND ISOCAPROIC ACIDS IN THE DIABETIC ORGANISM, WITH CONSIDERATION OF THE INTERMEDIARY METABOLISM OF LEUCINE AND VALINE.

By A. I. RINGER, E. M. FRANKEL AND L. JONAS.

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)

(Received for publication, April 30, 1913.)

For the proper understanding of the processes involved in the metabolism of the different foodstuffs it is essential to know the fate of the individual products of metabolism in the body. For the past few years this question has been the center of attack from different angles. The difficulties associated with these researches appeared insurmountable at times, but with the latest developments in the application of organic chemistry to biological processes more and more light is being thrown on the subject of the intermediary metabolism of foodstuffs.

There are a number of methods that have been devised of late for the prosecution of these researches, and while it must be admitted that every one of them may be associated with a certain amount of error, they have nevertheless all helped to bring to light certain definite reactions. The entire subject has been recently reviewed² and much space need not be given here to this phase of the problem.

In these researches animals were made diabetic by daily injections of 1 gram of phlorhizin ground up in a mortar with 10 cc. of olive oil. The urine was collected by catheter and at the end of each period of twelve hours the bladder was washed three times with warm distilled water.

¹ Aided by a grant from the Rockefeller Institute for Medical Research.

² Dakin: *Oxidations and Reductions in the Animal Body*, Longmans, Green and Company, London, 1912.

The urine was analyzed for its optical activity, nitrogen, glucose, ammonia, acetone, aceto-acetic acid and β -hydroxybutyric acid. In these determinations we were assisted by Mr. C. A. Hornberger.

The original purpose of these researches was to find the chemical configuration that a substance required in order to become converted into glucose in the diabetic organism. It soon became evident that the study of this problem was so closely interwoven with questions of the intermediary metabolism of foodstuffs and substances chemically related to them, that it was deemed necessary to extend the scope of this investigation to a broader field.

The method employed in these researches permits of the study of the following problems:

I. The fate of different substances in the animal body with reference to their ability to form glucose.

II. The fate of different substances with reference to their ability to give rise to β -hydroxybutyric acid, aceto-acetic acid and acetone.

III. The chemical changes that different substances undergo in the process of catabolism in the animal body before their conversion into glucose or acetone bodies.

IV. The influence of different substances on the metabolic processes of the diabetic organism—especially antiketogenesis.

V. The rôle of different substances in the pathology of diabetes.

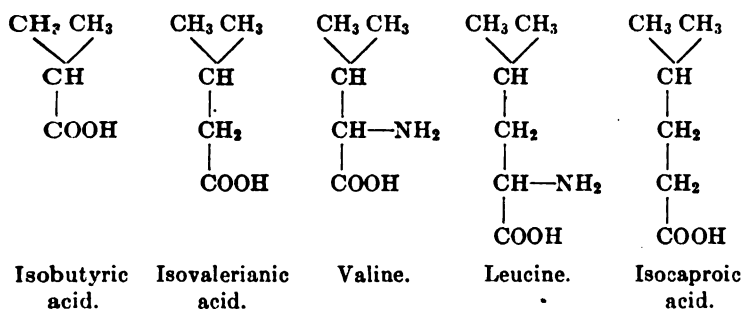
In the first two papers of this series it was shown that the normal fatty acids in the processes of catabolism in the diabetic animal undergo very definite reactions, by becoming oxidized in the β -position, giving rise to an acid with two carbons less. This conclusion was reached after having established the fact that propionic acid (when fed to diabetic dogs) is completely converted into glucose, and that of the higher fatty acids only those give rise to glucose which stand in relationship to propionic acid by having two (or a multiple thereof) carbons more. Thus *n*-valerianic and *n*-heptylic acids, containing five and seven carbons respectively, give rise to glucose, whereas *n*-butyric and *n*-caproic acids, containing four and six carbons respectively, do not give rise to glucose, but to an increase in the acetone bodies. These findings add increased support to Knop's hypothesis of β -oxidation.

In this communication are recorded the results of experiments

with isobutyric, isovalerianic and isocaproic acids fed to diabetic dogs.

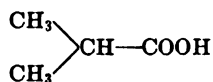
The methods employed were essentially the same as those of the previous experiments, with the exception that the phlorhizin was administered according to the Coolen method. It yields very satisfactory results, and with greater comfort to both operator and animal. The β -hydroxybutyric acid was determined by Shaffer's method.

The iso compounds enumerated above stand in very close relationship to several important amino-acids,



and the results of our experiments seem to throw light on the intermediary metabolism of these amino-acids.

The effect of isobutyric acid.



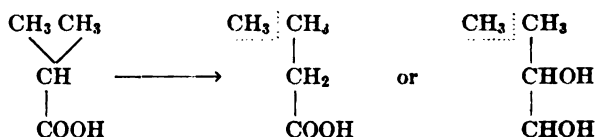
Baer and Blum³ gave 20 grams of isobutyric acid to a diabetic patient with the object of ascertaining whether it would cause increased elimination of the acetone bodies. They did not get any increase in the β -hydroxybutyric acid or acetone. Embden, Salomon and Schmidt⁴ perfused the liver with blood containing isobutyric acid and found no increase in the acetone formation.

In this series of experiments isobutyric acid was fed with a double object in view. First, both Baer and Blum and Embden

³ Baer and Blum: *Arch. f. exp. Path. u. Pharm.*, lv, p. 89, 1906.

⁴ Embden, Salomon and Schmidt: *Hofmeister's Beiträge*, viii, p. 129.

suggested the possibility of demethylation in the catabolism of the isobutyric acid molecule, with the introduction of either an H or an OH in the α -carbon:



If this were true, isobutyric acid would give rise to propionic acid or lactic acid, either of which should give rise to extra glucose. Second, it was of great importance to study the possible effects of isobutyric acid on acidosis.

In experiment XI, period VI, the animal was given subcutaneously 10 grams of isobutyric acid as sodium salt. The glucose elimination rose from 11.5 to 14.03 grams. The nitrogen elimination sank from 3.6 in the fore period to 3.29 and 2.55 in periods VI and VII, respectively, to rise again to 3.61 in period VIII. The amount of extra glucose eliminated was 5.52 grams (calculated by the method suggested by Lusk and Ringer).⁵ The most remarkable effect of the isobutyric acid was on the acetone and β -hydroxybutyric acid elimination. In the fore period the acetone elimination was 0.223 gram; it dropped down to 0.059 and rose again to almost the original level in period VII. The β -hydroxybutyric acid, which stood at 1.1 grams for two successive periods, dropped to 0.238 gram and returned to 1.325 grams as the effect of the isobutyric acid wore off. In period XII of the same experiment a similar dose of isobutyric acid was administered to the same dog. The effects were essentially the same, except that the amount of extra glucose was less than in the first case.

In periods IX and XII of experiment XII the animal received 10 grams of isobutyric acid. The yield of extra glucose was 7.6 grams in period IX and 4.3 grams in period XII.

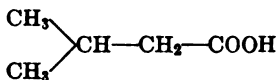
In period VI of experiment XII the animal received 20 grams of isobutyric acid, which resulted in the elimination of 9.15 grams of extra glucose. In period XII of the same experiment the animal was given subcutaneously 10 grams of isobutyl alcohol, which resulted in the elimination of 10.3 grams of extra glucose.

⁵ Ringer and Lusk: *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

All these experiments prove beyond question that *isobutyric acid gives rise to considerable quantities of extra glucose*. In most of the cases there was a very marked diminution in the nitrogen output, immediately following the isobutyric acid administration. This phenomenon is very marked in experiments XI, XII and XIV. It is, however, absent in experiment XIII, periods VI and VII.

Another point of great interest is the effect of isobutyric acid on the elimination of acetone bodies. In experiment XI the effect was very marked, while in the others it was hardly noticeable. These individual differences point very strongly to the existence of certain factors in the metabolism of the diabetic organism which determine the action of isobutyric acid and similar substances. Since these experiments were performed, we have administered isobutyric acid to several patients, some with very severe and others with moderately severe acidosis. It was found that in severe cases of diabetes most of the isobutyric acid is converted into glucose, no effect whatsoever being exerted on the acidosis. In the milder cases it is similarly converted into glucose, but very distinct anti-ketogenetic effects are noted. The influence of isobutyric acid on the nitrogen metabolism in animals and on acidosis in human diabetes will form the subject of a separate communication in the near future.

The effect of isovalerianic acid.



That isovalerianic acid gives rise to large quantities of β -hydroxybutyric acid, aceto-acetic acid and acetone has been proven beyond question by Baer and Blum⁶ in the diabetic patient and by Embden⁷ who found an increase in the aceto-acetic acid formation on perfusing an excised liver with blood containing 2 grams of isovalerianic acid.

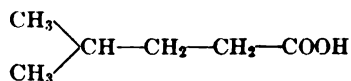
In this research it was our object to see whether isovalerianic acid has any influence on the glucose elimination. In experiment XI period IX, in experiment XII period VI and in experiment XIV

⁶ *Loc. cit.*

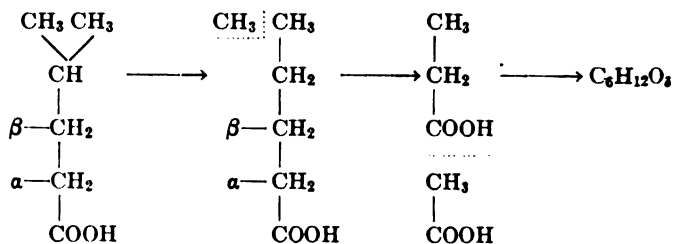
⁷ *Loc. cit.*

period XI, 10 grams of isovalerianic acid as sodium salt were administered subcutaneously. In none of these experiments was there any increase in the glucose. The elimination of the acetone bodies was increased to a remarkable extent, thus confirming the findings of Baer and Blum and of Embden.

The effect of isocaproic acid (isobutyl acetic acid).



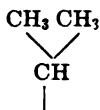
Embden perfused a dog's liver with blood containing isocaproic acid and obtained no increase in the aceto-acetic acid formation. We performed our experiments with this substance to see what influence it has on acidosis and whether it will give rise to extra glucose. Reasoning *a priori*, one would expect this substance to give rise to glucose. The process of demethylation having been established in iso compounds in the case of isobutyric and isovalerianic acids, there is every reason to suppose that such a process takes place in the isocaproic acid molecule. Valerianic acid would be formed first, which after undergoing β -oxidation, would give rise to propionic acid and to glucose.



In period III of experiment XIII, 11.6 grams of isocaproic acid were given subcutaneously as sodium salt. The glucose elimination rose from 24.6 grams in the fore period to 25.19 grams, in spite of the drop in the nitrogen. The D:N ratio, which was 3.38 in the fore period and 3.36 in the after period, rose to 3.65. The extra glucose eliminated was 1.95 grams. In experiments XIV periods XVIII and XIX and in experiment XV period IX, 10 grams of isocaproic acid as sodium salt were given *per os*. The results in both cases corroborate the results in the first experiment in showing

that extra glucose is formed from isocaproic acid. It is true that the amount of extra glucose does not come up to the theoretical value, but, as will be seen from other experiments, theoretical yields are not always obtained.

Intermediary metabolism of fatty acids containing an isopropyl radical.



From the work of Baer and Blum, Embden and our own, it is apparent that all compounds containing an isopropyl radical undergo demethylation. The question of interest in this connection is, in what way does the methyl radical dissociate from the rest of the molecule? Baer and Blum have advanced the suggestion that the CH_3 may leave the molecule and an OH radical take its place. They based this suggestion upon the fact that after feeding 20 grams of isobutyric acid they obtained 0.5 gram of zinc lactate from one-half of the twenty-four hours' urine, and also upon the fact that isovalerianic acid gave rise to β -hydroxybutyric acid. We are inclined to believe that the methyl radical is removed by a process of hydrolysis, the OH going to the methyl radical, forming CH_3OH , which is oxidized in the body, while the H radical goes to replace the CH_3 , forming an acid of the normal series. Thus isobutyric acid gives rise to propionic acid which in turn may give rise to lactic acid. Isovalerianic acid gives rise to butyric acid which in turn produces β -hydroxybutyric acid. This also explains why Baer and Blum obtained β -hydroxybutyric acid from α -methylbutyric acid and failed to get it from α -hydroxybutyric acid.

Intermediary metabolism of amino-acids containing an isopropyl radical.

Embden and Marx⁸ in their perfusion experiments found that α -amino-valerianic acid gave rise to aceto-acetic acid, while neither α -amino-butyric nor α -amino-caproic acids showed any evidence of

⁸ Embden and Marx: *Hofmeister's Beiträge*, xi, p. 318, 1908.

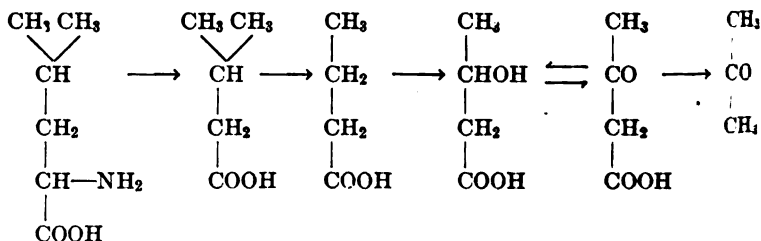
yielding aceto-acetic acid. They drew the conclusion that α -amino-acids suffer oxidation in the α -position giving rise to acids with one carbon atom less, while the carboxyl radical is split off, in all probability in the form of CO_2 .

In the protein molecule we find two amino-acids which contain the isopropyl radical—leucine and valine. Halsey* fed leucine to phlorhizinized dogs, and, of six feedings, two showed considerable amounts of extra glucose, and four gave entirely negative results. Recently Dakin published one experiment in which he fed 15 grams of *i*-leucine and obtained 4 grams of extra glucose. Neither Halsey nor Dakin are inclined to attribute glucogenetic properties to leucine.

When we consider leucine in the light of our experiments with unsubstituted fatty acids with branched chains, and having in mind Embden's results, in which he showed that leucine is a strong aceto-acetic acid builder, there remains only one possible conclusion with regard to the catabolism of leucine. As far as our knowledge goes, and as will be shown in a following communication, we are fully justified in assuming that all carbons in the α -position having an amino radical lose the amino radical and become oxidized possibly to the carboxyl state, giving rise to compounds (acids) with one carbon less.

For the present we do not include glycocoll or alanine in this category. Experiments are in progress which will throw light on the rôle of the carboxyl in the formation of glucose from these substances.

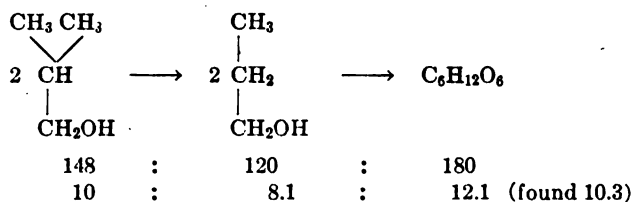
Leucine in its catabolism can be assumed to pass through the following intermediary stages:



* Halsey: *Amer. Journ. of Physiol.*, x, p. 229, 1903.

Ordinarily leucine cannot be classed as a glucose-yielding substance, but under certain circumstances it may give rise to glucose. There are certain flora of intestinal bacteria that seem to possess the power of effecting α -oxidation. In one of our experiments butyric acid, which, when given subcutaneously never yields even a trace of glucose, when fed *per os* gave rise to as much as 3 grams of glucose. This reaction cannot be explained in any other way, and it seems very possible that the same factors obscured Halsey's two experiments.

When we found that isobutyric acid gave rise to glucose and that isobutyl alcohol could yield an amount of glucose that corresponds to the conversion of three of its carbons into glucose, we were led to the belief that valine in its catabolism was broken down to isobutyric acid, and that it would be found to be one of the amino-acids that give rise to glucose.



In his paper on the "Intermediary Metabolism of Amino-acids," Dakin¹⁰ showed that valine yields practically no glucose. This of course makes our theory more difficult of interpretation, but we do not believe it robs the theory of its strength, for the antiketogenic properties of valine are very similar to those of isobutyric acid, and as will be shown in the future, this property is possessed only by compounds capable of forming glucose. (The reverse, however, is not true, *i.e.*, not all compounds that are capable of forming glucose possess antiketogenic properties.)

SUMMARY.

Experiments were performed on phlorrhizinated dogs.

I. It was found that isobutyric acid and isobutyl alcohol give rise to glucose, probably by undergoing demethylation and by giving rise to normal fatty acids (propionic acid).

¹⁰ Dakin: This *Journal*, xiv, p. 321, 1913.

II. Isovalerianic acid does not give rise to glucose, but to large quantities of aceto-acetic acid, acetone and β -hydroxybutyric acid.

III. Isocaproic acid was found to give rise to glucose, probably having, by a process of demethylation, formed normal valerianic acid, which became oxidized to propionic acid.

IV. Isobutyric acid, in certain cases, possesses very marked anti-ketogenetic properties.

V. It is suggested that isovalerianic acid is one of the intermediary stages in the catabolism of leucine.

VI. It is also suggested that isobutyric acid may be an intermediary body in the catabolism of valine.

EXPERIMENT XI. Twelve-hour periods.

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D:N	EXTRA GLUCOSE	NH ₄ N	ACETONE AND ACETIC ACID	β -HYDROXY-BUTYRIC ACID	REMARKS
Feb.											
27	III		3.96	13.76	+0.63°	3.47		0.153	0.128	0.509	1 gm. phlorhizin.
27	IV	8.66	3.45	11.21	+0.50°	3.26		0.226	0.197	1.12	
28	V		3.60	11.50	+0.52°	3.20		0.274	0.223	1.165	1 gm. phlorhizin.
28	VI	8.32	3.29	14.03	+0.66°	4.26		0.187	0.059	0.238	10 gms. isobutyric acid given subcutaneously as Na salt in 2 doses.
March							5.52				
1	VII		2.55	10.44	+0.45°	4.11		0.134	0.210	1.325	1 gm. phlorhizin.
1	VIII	8.26	3.36	11.05	+0.46°	3.29		0.266			
2	IX		3.61	11.47	+0.44°	3.18		0.334	0.549	2.75	1 gm. phlorhizin. 10 gms. isovalerianic acid.
2	X	8.08	3.24	10.75	+0.41°	3.32		0.218	0.37	2.50	
3	XI		3.31	10.52	3.18			0.266	0.183	1.14	1 gm. phlorhizin.
3	XII		3.18	12.42	+0.54°	3.91	2.55	0.100	0.059	0.237	10 gms. isobutyric acid dissolved in 30 cc. of water; given subcutaneously as Na salt in 2 doses.
4	XIII		2.60	7.88	3.03			0.157	0.076	0.671	1 gm. phlorhizin.
4	XIV	7.82	2.30	7.62	3.31			0.142	0.219	1.29	
5	XV		2.68	8.35	3.11			0.204	0.262	1.57	1 gm. phlorhizin.

Chemistry of Gluconeogenesis

EXPERIMENT XII. Twelve-hour periods.

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D: N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO- ACID	β -HYDROXY- BUTYRIC ACID	REMARKS
Feb.											
27	III		5.14	18.93	+0.85°	3.69		0.555	0.211	0.830	1 gm. phlorhizin.
27	IV	12.70	4.60	16.90	+0.76°	3.68		0.434	0.287	1.508	
28	V		5.02	17.51	+0.765°	3.49		0.498	0.328	2.44	1 gm. phlorhizin.
28	VI	12.30	3.98	15.55	+0.53°	3.91		0.541	0.037	5.81	10 gms. isovalerianic acid.
March											
1	VII		4.83	17.86	+0.75°	3.70		0.714	0.574	3.58	1 gm. phlorhizin.
1	VIII	12.30	4.30	16.37	+0.72°	3.81		0.565	0.410	1.95	
2	IX		3.28	17.50	+0.73°	5.34		0.841	0.281	1.80	1 gm. phlorhizin. 10 gms. isobutyric acid, subcutaneously.
	X	12.38	3.09	14.85	+0.68°	4.82	7.60	0.230	0.305	1.29	
3	XI		3.74	14.84		3.96		0.470	0.008	2.16	1 gm. phlorhizin.
3	XII	12.08	2.93	13.86	+0.625°	4.75		0.180	0.642	1.05	10 gms. isobutyric acid given subcutaneously as Na salt in 2 doses.
	XIII		2.96	12.20		4.13	4.31	0.174	0.464	1.50	1 gm. phlorhizin.
4	XIV	11.66	3.44	11.70		3.43		0.214	0.370	1.445	
5	XV		3.34	10.40		3.14		0.332	0.454	1.74	1 gm. phlorhizin.

EXPERIMENT XIII. *Twelve-hour periods.*

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZA- TION	D: N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO- ACID	β -HYDROXY- BUTYRIC ACID	REMARKS
March											
12	II		7.28	24.60	+1.158°	3.38		0.587	0.311	2.182	1 gm. phlorhizin.
13	III	15.30	6.90	25.19	+1.20°	3.65	1.95	0.308	0.259	1.087	11.6 gms. isocaproic acid given subcu- taneously as Na salt.
13	IV		7.14	23.95	+1.15°	3.36		0.383	0.233	0.922	1 gm. phlorhizin.
14	V	15.00	6.98	22.26	+0.96°	3.19		0.505	0.297	1.254	
14	VI		6.87	27.90	+1.30°	4.06	9.15	0.278	0.299	1.43	1 gm. phlorhizin. 20 gms. isobutyric acid given subcutaneously as Na salt in 2 doses.
15	VII		7.27	25.65	+1.21°	3.53		0.210	0.320	0.821	
15	VIII	14.52	7.61	23.48	+1.02°	3.09		0.415	0.350	0.924	1 gm. phlorhizin.
16	IX		6.87	21.18	+1.07°	3.07		0.325	0.347	0.992	
16	X		5.98	23.09	+0.90°	3.86		0.292	0.280	0.604	1 gm. phlorhizin.
17	XI	13.72	5.25	20.14	+0.86°	3.84		0.265	0.149	0.383	
17	XII		4.50	21.38	+0.86°	4.75	10.28	0.293	0.135	0.477	1 gm. phlorhizin. 10 gms. isobutyl alco- hol given subcutaneously.
18	XIII	13.12	4.09	20.40	+0.905°	5.00		0.366	0.185	0.542	
18	XIV		5.74	20.09	+0.86°	3.50		0.364	0.178	0.520	1 gm. phlorhizin.

EXPERIMENT XIV. Twelve-hour periods.

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D: N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO-ACID	β -HYDROXY-BUTYRIC ACID	REMARKS
March											
16	X		4.93	19.45	+0.83°	3.95		0.253	0.219	0.55	1 gm. phlorhizin.
17	XI	11.20	5.42	18.55	+0.77°	3.42		0.359	0.55	2.39	10. gms. isovaleric acid given subcutaneously as Na salt in 1 dose.
17	XII		5.61	18.59	+0.78°	3.31		0.346	0.455	2.58	1 gm. phlorhizin.
18	XIII	10.68	6.46	21.46	+0.895°	3.35		3.342	0.314	1.66	
18	XIV		5.80	19.70	+0.805°	3.40		0.439	0.330	2.24	1 gm. phlorhizin.
19	XV		5.17	20.67	+0.875°	4.00	3.21	0.252	0.273	1.79	10 gms. isobutyric acid given <i>per os</i> as Na salt.
19	XVI*	{	4.78	16.05	+0.613°	3.36		0.515	0.435	2.87	1 gm. phlorhizin.
20			4.78	16.05	+0.613°	3.36		0.515	0.435	2.87	
20	XVII		10.22	4.73	+0.615°	3.16		0.605	0.331	1.93	1 gm. phlorhizin.
21	XVIII		3.75	12.24	+0.565°	3.27	2.78	0.270	0.303	1.29	10 gms. isocaproic acid given <i>per os</i> as Na salt.
21	XIX		9.69	3.45	+0.512°	3.85			0.333		1 gm. phlorhizin.

* Analysed together.

EXPERIMENT XV. Twelve-hour periods.

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D: N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO-ACID	β -HYDROXY-BUTYRIC ACID	REMARKS
March											
30	VIII	7.10	2.80	9.45	+0.378°	3.37			0.540	1.935	1 gm. phlorhizin given subcutaneously.
1	IX		2.83	10.51	+0.380°	3.71			0.705	2.060	10 gms. isocaproic acid given <i>per os</i> as Na salt.

THE CHEMISTRY OF GLUCONEOGENESIS.¹

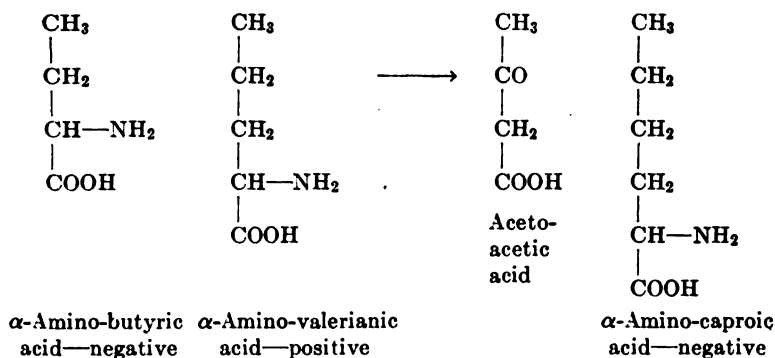
IV. THE FATE OF SUCCINIC, MALIC AND MALONIC ACIDS IN THE DIABETIC ORGANISM, WITH CONSIDERATION OF THE INTERMEDIARY METABOLISM OF ASPARTIC AND GLUTAMIC ACIDS, PROLINE, LYSINE, ARGININE AND ORNITHINE.

By A. I. RINGER, E. M. FRANKEL AND L. JONAS.

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)

(Received for publication, April 30, 1913.)

Embden and Marx² found that on perfusing an extirpated liver with blood to which α -amino-valerianic acid had been added, there was an increase in the aceto-acetic acid formation, while α -amino-butyric acid and α -amino-caproic acid gave negative results.



It is evident that α -amino-valerianic acid must have been changed to a four-carbon compound before it could possibly give rise to aceto-acetic acid. The authors therefore concluded that in α -amino-acids the α -carbon, containing the amino radical, becomes

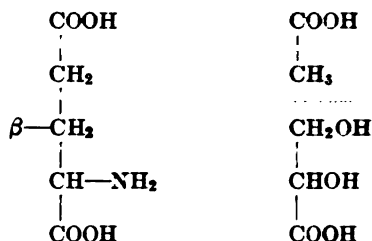
¹ Aided by a grant from the Rockefeller Institute for Medical Research.

² Embden and Marx: *Hofmeister's Beiträge*, xi, p. 318, 1908.

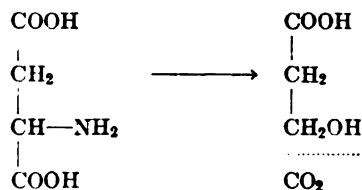
oxidized to a carboxyl state, giving rise to a fatty acid with one carbon less.

Lusk³ found that the feeding of 20 grams of glutamic acid was followed by an elimination of 13.5 grams of extra glucose.

Ringer and Lusk⁴ extended these investigations and found that 20 grams of aspartic acid yielded as much as 14.9 grams of extra glucose. These results corresponded to the conversion into glucose of three of the carbons of either the aspartic or glutamic acid molecule. It was then suggested that glutamic acid undergoes the following changes in the diabetic organism by giving rise to glyceric acid, which was found capable of forming extra glucose.



For aspartic acid it was suggested that the molecule may suffer oxidation in the α -position, i.e., in the carbon containing the amino radical, giving rise to hydracrylic acid.

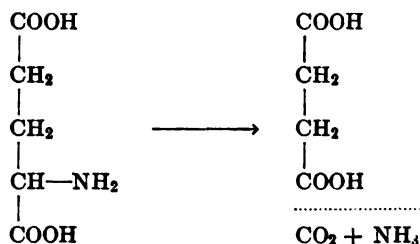


In this series of researches it was our object to test experimentally the paths that these amino-acids may take in their catabolism. In view of the outcome of Embden and Marx' experiments, it suggested itself that glutamic acid may undergo deaminization with oxidation in the α -carbon giving rise to a

³ Lusk: *Amer. Journ. of Physiol.*, xxii, p. 174, 1908.

⁴ Ringer and Lusk: *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

dibasic acid with one less carbon, *i.e.*, with four carbons, namely, succinic acid. If this were true, succinic acid would yield glucose in the diabetic animal as readily as does glutamic acid.



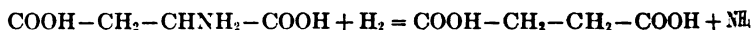
In experiment XVI, period IV, 11.8 grams ($\frac{M}{10}$) of succinic acid as sodium salt were given *per os*. The glucose elimination, which was 27.26 in the fore period and 25.02 in the after period, rose to 32.74 grams. The D:N ratio rose from 3.41 to 3.99. The amount of extra glucose eliminated was 5.15 grams. In experiment XVII, period XIII, 11.8 grams of succinic acid as sodium salt were given subcutaneously. The yield of extra glucose was much larger, amounting to 9.45 grams.

These two experiments prove very conclusively that succinic acid can yield large quantities of extra glucose. The question now arises—is it merely an incident that these two substances give rise to glucose or does it actually *prove* that glutamic acid passes through succinic acid in its intermediary stages of metabolism? We believe that the latter is the case, and that glutamic acid *does* give rise to succinic acid for the following additional reason: Succinic acid is found as one of the by-products of alcoholic fermentation. Pasteur⁵ proved its presence conclusively and found that the quantity of succinic acid bore a relationship of 0.4 to 0.7 per cent to the fermented glucose. He believed that succinic acid was a product of the fermentation of the glucose molecule. This theory of Pasteur greatly disturbed the then current conception of Gay-Lussac that a molecule of glucose, in the process of fermentation, breaks down to two molecules of alcohol and two molecules of carbon dioxide.



⁵ Pasteur: *Compt. rend. Acad. Sci.*, 1858 to 1859; *Ann. de chim. et de phys.*, (3) lviii, p. 323, 1860.

During the next forty years this was the subject of a great many investigations and slowly evidence accumulated which tended to disprove Pasteur's theory of succinic acid arising from the glucose molecule. It was shown that the quantity of succinic acid was not proportional to the amount of glucose fermented, but to the length of time that fermentation was permitted to go on. Buchner and Meisenheimer⁶ finally succeeded in showing that succinic acid was not a product of glucose fermentation, but a product of the metabolism of the yeast cell. Working with expressed cell-free juice of yeast, they could find no succinic acid. The problem of the origin of succinic acid in the by-products of alcoholic fermentation was solved definitely by Felix Ehrlich.⁷ He found that the higher alcohols of fusel oil were the products of the protein metabolism of the yeast cell. He then devoted his researches to finding the mother substances of succinic acid. It was already known at that time that bacteria were capable of effecting deamination of amino-acids by splitting off the NH_2 radical and converting it into NH_3 substituting a hydrogen for the removed NH_2 .



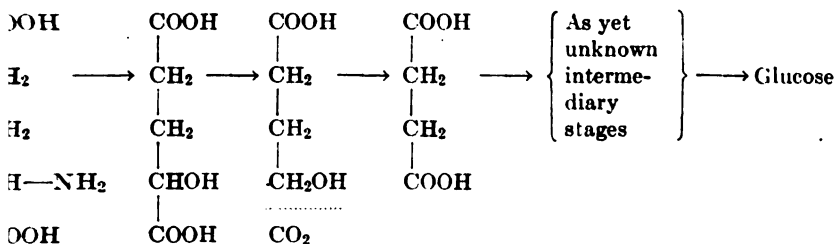
It was also known that in addition to succinic acid small quantities of aspartic acid were found in the fermentation mixture. It therefore suggested itself to Ehrlich that aspartic acid might be the mother substance of succinic acid and that glutamic acid might be the mother substance of glutaric acid, which would mean, if proven, that yeast cells, like bacteria, are capable of bringing about deamination in a very simple way—by splitting off NH_2 and substituting a hydrogen for it. When he came to subject these ideas to the test of experimentation, he found that the addition of aspartic acid to fermenting yeast and sugar was followed by no increase in the succinic acid, but when glutamic acid was added, he was able "*stets sehr beträchtliche, den normalen Gehalt weit übersteigende Mengen von Bernsteinsäure, zu isolieren.*"

Here we see the direct transformation of glutamic acid into succinic acid by a living cell, and when we compare these results

⁶ Buchner and Meisenheimer: *Ber. d. deutsch. chem. Gesellschaft.*, xxxix, p. 3201, 1906; xxxiv, p. 1529, 1901.

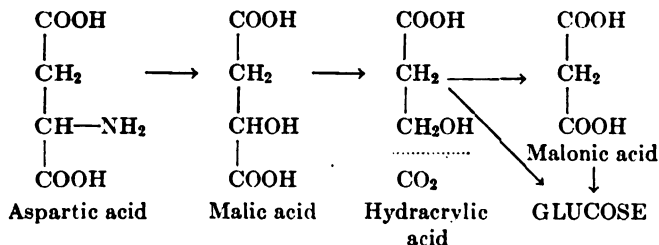
⁷ Ehrlich: *Biochem. Zeitschr.*, xviii, p. 391, 1909.

with our own in the diabetic organism, we feel fully justified in concluding that *the path of glutamic acid in metabolism is through succinic acid undoubtedly passing through α -hydroxy-glutaric acid and γ -hydroxy-butyric acid as intermediary stages:*



The fate of aspartic acid in the diabetic organism.

In the foregoing it was shown that α -amino-glutaric acid (glutamic acid) becomes catabolized to succinic acid, which in turn gives rise to extra glucose. A similar path of catabolism suggests itself for aspartic acid, which is chemically very closely related to glutamic acid.



Aspartic acid, as was shown by Ringer and Lusk,⁸ and asparagine, as was shown by Knopf,⁹ can give rise to large quantities of glucose. In tracing the possible intermediary compounds, one should find all of them capable of yielding glucose to the same extent as does aspartic acid. The first intermediary product of aspartic acid after deaminization appears to be malic acid. In experiment XVI, period II, 13.4 grams ($\frac{M}{10}$) of malic acid as sodium salt were administered subcutaneously. The amount of glucose

⁸ Ringer and Lusk: *loc. cit.*

⁹ Knopf: *Arch. f. exp. Path. u. Pharm.*, xlix, p. 123, 1903.

elimination rose considerably, but not all of it can be attributed to the malic acid, because there was a considerable rise in the protein metabolism of the same period. The D:N ratio rose from 3.4 in the fore period to 3.97 in the experimental period. The amount of extra glucose eliminated was 5.94 grams. Very convincing results were obtained in experiment XX, period VII. 13.4 grams of malic acid, neutralized with calculated amounts of sodium and potassium hydroxide, were given *per os*. The glucose elimination rose from 14 grams in the fore period to 21.14 grams in spite of the drop of the nitrogen metabolism. The D:N ratio rose from 3.12 to 5.27 and returned in the after periods to 3.27 and 3.22. The calculated extra glucose amounted to 8.32 grams.

These two experiments prove very conclusively that malic acid gives rise to glucose to approximately the same extent as does aspartic acid, and that it may be considered a product of aspartic acid metabolism.

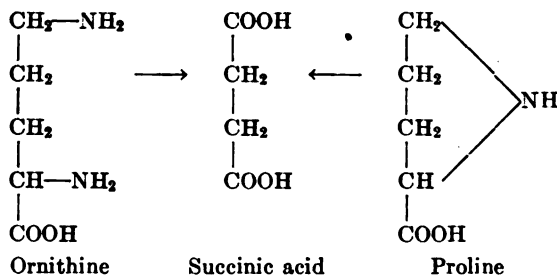
The next question to decide was whether hydracrylic acid and malonic acid give rise to glucose. Unfortunately we had none of the former in our possession and were forced to postpone the experiment to a future date. Malonic acid was administered seven times to five different dogs. It was found that because of the large quantities of alkali necessary to neutralize the acid, it was absorbed with great difficulty, and we were never sure that all of the administered material was absorbed. In some cases it was administered *per os*, in others subcutaneously. The oral administrations were always followed by diarrhoea. The amounts of extra glucose produced by malonic acid are here tabulated:

Experiment	XVII	Period	II	Extra Glucose	0.00	grams.
"	XVIII	"	XVI	"	"	1.00 "
"	XIX	"	IV	"	"	2.64 "
"	XIX	"	VII	"	"	0.65 "
"	XIX	"	X	"	"	2.84 "
"	XX	"	III	"	"	3.06 "
"	XXI	"	III	"	"	1.00 "

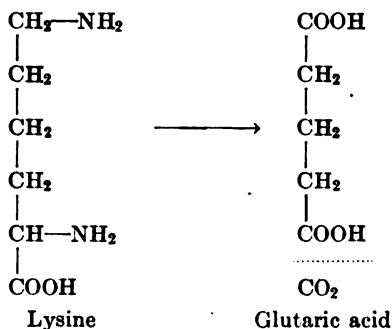
The results are very low indeed, but it is very likely that in the transformation of aspartic acid to a compound with three carbons, the reaction ordinarily does not proceed to a point of complete oxidation of the terminal carbon. It is very likely that hydracrylic acid goes over into glucose before the alcohol radical becomes oxidized to a carboxyl.

Intermediary metabolism of lysine, arginine, ornithine and proline.

In a recent series of papers, Dakin¹⁰ showed that arginine, ornithine and proline give rise to glucose, when fed to diabetic dogs, while lysine does not give rise to glucose. Dakin rightly suggests that of the arginine only the ornithine moiety goes over into glucose. It seems to us that ornithine and proline give rise to glucose because of their ability to form succinic acid after undergoing deamination.



Lysine, on the other hand, after undergoing deamination, becomes converted into glutaric acid and this was shown not to be convertible into glucose.



SUMMARY.

Experiments were performed on phlorhizinized animals.

I. It was found that succinic, malic and perhaps also malonic acids give rise to extra glucose.

¹⁰ Dakin: this *Journal*, xiii, p. 513, 1913; xiv, p. 321, 1913.

II. Evidence was presented to the effect that succinic acid is an intermediary body in the metabolism of glutamic acid, ornithine and proline, which accounts for the conversion of these substances into glucose.

III. It is suggested that malonic acid may arise in part from the catabolism of aspartic acid.

IV. It was also suggested that lysine in its catabolism passes through a glutaric acid stage, which accounts for its non-conversion into glucose.

EXPERIMENT XVI. Twelve-hour periods.

DATE	WEIGHT	PERIOD	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZA- TION	D: N	EXTRA GLUCOSE	NH ₃ N	TOTAL ACETONE	REMARKS
Oct. 1912										
30		I	8.35	28.37	1.096°	3.40		0.56	0.28	
30	14.49	II	10.29	40.92	1.635°	3.97	5.94	0.29	0.33	13.4 gms. malic acid as sodium salt dissolved in water given subcutaneously.
31		III	7.99	27.26	1.150°	3.41		0.33	0.25	
31		IV	8.19	32.74	1.402°	3.99	5.15	0.20		11.8 gms. succinic acid as sodium salt given per os.
Nov. 1		V	7.49	25.02		3.34		0.37	0.367	

EXPERIMENT XVII. Twelve-hour periods.

DATE	WEIGHT	PERIOD	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZA- TION	D: N	EXTRA GLUCOSE	NH ₃ N	TOTAL ACETONE	REMARKS
Nov. 1912										
14	12.90	I	3.72	14.38		3.86		0.19	0.56	
14		II	4.20	14.94		3.53	0.0	0.24	0.95	10.4 gms. malonic acid as sodium salt dissolved in water given subcutaneously.
15		III	3.90	14.79		3.79		0.22	0.35	

EXPERIMENT XVIII. Twelve-hour periods.

DATE	WEIGHT	PERIOD	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D: N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETIC ACID	β -HYDROXY-BUTYRIC ACID	REMARKS
April 1913											
7		I	6.14	17.85	0.80°	2.92		0.53			
7	12.53	II	5.53	16.84	0.85°	3.05		0.46	0.47	1.16	
8		III	5.35	24.30	1.13°	4.55	9.45	0.25	0.41	1.14	11.8 gms. succinic acid as sodium salt dissolved in water, given subcutaneously.
8	12.46	IV	4.47	16.87	0.80°	3.78		0.28	0.37	1.46	
9		V	4.53	15.43	0.72°	3.41		0.33	0.48	1.73	
9	12.71	VI	4.20	16.96	0.78°	3.80	1.00	0.30	0.45	1.86	10.4 gms. malonic acid as sodium salt given per os. Diarrhoea.
10		VII	3.05	11.21	0.62°	3.68		0.22	0.41	1.56	
10	12.32	VIII	3.11	11.76	0.50°	3.78			0.56	2.34	

EXPERIMENT XIX. Twelve-hour periods.

DATE	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D:N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO-ACID	β -HYDROXY-BUTYRIC ACID	REMARKS
April 1913											
6	I		4.34	14.22	0.625°	3.28		0.27	0.15	0.39	10.4 gms. malonic acid as sodium salt given <i>per os</i> in one dose. One watery movement of bowels.
7	II		5.10	16.55	0.88°	3.24		0.30	0.22	0.54	
7	III		4.99	16.05	0.75°	3.24		0.28	0.25	0.79	
8	IV	6.80	5.21	19.82	0.87°	3.31	2.64	0.23	0.41	1.56	
8	V		5.08	17.36	0.80°	3.42		0.29	0.52	1.72	10.4 gms. malonic acid as sodium salt dissolved in water given subcutaneously.
9	VI	6.45	4.99	16.86	0.73°	3.38		0.33	0.48	1.87	
9	VII		4.93	17.45	0.78°	3.54	0.65	0.30	0.45	0.98	
10	VIII	6.43	4.67	16.05	0.73°	3.44		0.27	0.17	0.51	10.4 gms. malonic acid as sodium salt given <i>per os</i> . Diarrhoea.
10	IX		5.28	16.95	0.75°	3.21		0.29	0.17	0.52	
11	X	6.25	2.88	11.83	0.55°	4.11	2.84	0.13	0.12	0.30	
11	XI		3.79	11.46	0.52°	3.03		0.17	0.21	0.63	
12	XII		3.39	9.60		2.83		0.32	0.10	0.26	

EXPERIMENT XX. Twelve-hour periods.

DATE	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D: N	EXTRA GLUCOSE	NH ₄ N	TOTAL ACETONE	PERFORMED ACETONE	ACETO-ACETIC ACID	β -HYDROXY-BUTYRIC ACID	REMARKS
April 1913													
14	I	13.40	7.99	29.35	1.26°	3.67		0.46	0.310	0.061	0.249	1.01	10.4 gms. malonic acid as sodium and potassium salt given subcutaneously in two doses.
15	II	8.13	30.41	1.39°	3.74			0.52	0.365	0.059	0.306	1.28	
15	III	13.09	8.20	33.86	1.50°	4.13	3.06	0.53	0.466	0.061	0.406	1.73	
16	IV		9.08	34.39	1.60°	3.79		0.58	0.457	0.083	0.374	1.74	
16	V	12.91	9.42	36.38	1.62°	3.86		0.64	0.554	0.028	0.526	2.39	
17	VI		7.49	28.25	1.32°	3.78		0.47					

EXPERIMENT XXI. Twelve-hour periods.

DATE	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D: N	EXTRA GLUCOSE	NH ₄ N	TOTAL ACETONE	PERFORMED ACETONE	ACETO-ACETIC ACID	β -HYDROXY-BUTYRIC ACID	REMARKS
April 1914													
15	II		4.43	12.50	0.60°	2.82		0.25	0.206	0.129	0.077		10.4 gms. malonic acid as sodium and potassium salt dissolved in water given subcutaneously in two doses.
15	III	10.39	4.93	15.64	0.73°	3.17	1.00	0.32	0.215	0.078	0.137	0.792	
16	IV		4.81	14.90	0.61°	3.10		0.32	0.283	0.087	0.196	1.32	
16	V	10.21	4.32	14.32	0.63°	3.32		0.36	0.271	0.081	0.190	1.86	
17	VI		4.50	14.00	0.60°	3.12		0.45	0.295	0.096	0.229	1.96	13.4 gms. malic acid as sodium and potassium salt given per os.
17	VII	9.94	4.02	21.14	1.04°	5.37	8.32	0.06	0.313	0.126	0.196	1.23	
18	VIII		3.90	12.92	0.49°	3.27		0.32	0.311	0.129	0.371	2.09	
18	IX	9.77	3.26	13.61	0.66°	3.24		0.41	0.560	0.394	0.201	3.24	

ON THE ACTION OF LEUCOCYTES ON HEXOSES.

FOURTH COMMUNICATION.

ON THE MECHANISM OF LACTIC ACID FORMATION.

BY P. A. LEVENE AND G. M. MEYER.

(From the Rockefeller Institute for Medical Research, New York.)

(Received for publication, April 30, 1913.)

It was reported in a previous communication that by the action of leucocytes on *d*-glucose, *d*-mannose or *d*-fructose, *d*-lactic acid was obtained. The mechanism of this transformation may be interpreted by a process consisting of at least three phases: First, transformation of the six carbon chain molecule into two three carbon chains such as glyceric aldehyde; second, into the formation of a substance which would permit a change in the configuration of the groups attached to the α -carbon atom (to the carbonyl group); and third, the transformation of the latter substance into lactic acid. It is evident that the formation of methyl glyoxal in the second phase of the reaction could lead the way to lactic acid formation by enzymes analogous to those which Knoop and Dakin found capable of transforming keto-acids into hydroxy-acids. At the moment when the previous communication was prepared for publication, evidence of the existence of such enzymes was lacking, hence the question was referred to as one requiring further experimental investigation. In fact, experiments in that direction were initiated immediately after the observations reported in the previous communication had been completed.

Simultaneously with our observation on the transformation of the three hexoses into *d*-lactic acid, Dakin and Dudley¹ made the very important discovery of the presence in animal tissues of an enzyme capable of transforming phenyl glyoxal into the natural mandelic acid and later they supplemented it by an observation on the transformation of methyl glyoxal into lactic acid.

¹ Dakin and Dudley: this *Journal*, xiv, p. 155, 1913.

The observations of Dakin and Dudley were soon corroborated by Neuberg,² who observed the transformation of pyruvic aldehyde into lactic acid. It seemed at first sight that these observations entirely established the correctness of the view of the mechanism of the transformation of hexoses into lactic acid expressed in our previous communication. However, the conclusion was not absolutely compelling for the following reasons.

First, under the conditions of the experiments of Dakin and Dudley and of Neuberg, hexoses are not converted into lactic acid, but remain intact, and it does not naturally follow that an enzyme present in autolyzing tissues also is present in the uninjured cells. Second, neither Dakin and Dudley nor Neuberg make positive statements regarding the absolute absence of bacterial growth in their solutions. The presence or absence of bacterial contamination is an all important factor for the consideration of the extent to which the observations of Dakin and Dudley and of Neuberg can be applied to the explanation of the mechanism of conversion of the *d*-hexoses into *d*-lactic acid. The enzymes active in the conversion of hexoses into lactic acid bring about the formation of only the dextro-rotatory substance. In the experiments of Neuberg a mixture of *dl* and of the *d* forms was obtained. It is not excluded, under such conditions, that by the action of the enzyme only the racemic acid was formed and that the dextro-rotatory isomer owed its origin to bacterial action. At the time of Dakin and Dudley's first communication the authors brought no evidence of the formation of the optically active acid. Third, in the work of Neuberg there is a lack of evidence that the *d*-lactic acid was not derived from the liver tissue employed in his experiment.

By all this we do not for a moment intend to detract from the importance of the discovery of Dakin and Dudley and of the observations of Neuberg. However, we felt that in order to establish our first view on the mechanism of lactic acid formation from hexoses beyond dispute, it was imperative to demonstrate the conversion of methyl glyoxal into *d*-lactic acid under the same conditions which enabled us to bring about the conversion of *d*-hexoses into *d*-lactic acid. For these reasons, and with the consent of Dr. Dakin.

² Neuberg: *Biochem. Zeitschr.*, xlix, p. 502.

we continued our experiments which were in progress at the time of his first publication.

Our experiments were performed under exactly the same conditions as those for the conversion of hexoses into lactic acid. There was an absolute absence of bacterial growth, both aerobic and anaerobic. Leucocytes and kidney tissue were employed in the experiments. The conversion of methyl glyoxal into lactic acid took place with either of the tissues. The lactic acid was a mixture of *dl* and of *d* forms. This furnishes convincing evidence to the view that the formation of *d*-lactic acid from the various *d*-hexoses is conditioned by the intermediate formation of methyl glyoxal.



Controls with leucocytes alone and with kidney tissue alone (in quantities equal to those employed in the principal experiments) showed the absence of lactic acid.

EXPERIMENTAL.

Leucocytes. These were obtained from dogs by injecting turpentine into the pleural cavity, according to the method previously described.

Tissues. Rabbits were killed by exsanguination and the kidneys removed aseptically, and minced as fine as possible under sterile conditions.

Methyl glyoxal. This was prepared from methyl glyoxal-diacetol according to Meisenheimer.³ The latter compound was obtained by the method of A. Wohl and M. Lange.⁴

Solutions. The methyl glyoxal (about 1 gram) was dissolved in 10 cc. of distilled water and sterilized by passing it through a Berkefeld filter. The washed leucocytes were suspended in a 1 per cent Henderson phosphate mixture to which had been added calcium phosphate, 3 grams to each 100 cc. of solution. The sterile methyl glyoxal solution was added last. The same procedure was adopted in the experiment with the kidneys.

Lactic acid. The mixture of methyl glyoxal with leucocytes

³ Meisenheimer: *Ber. d. deutsch. chem. Gesellschaft.*, xlv, p. 2635, 1912.

⁴ A. Wohl and M. Lange: *Ber. d. deutsch. chem. Gesellschaft.*, xli, p. 3612, 1908.

and kidneys was set aside at 37° for eighteen hours, and then coagulated by heating and with the addition of sufficient phosphoric acid to dissolve the calcium phosphate. The filtered solution was extracted with ether in a von der Heide extracting apparatus after the addition of sodium sulphate and 5 cc. of phosphoric acid. The zinc salt was prepared according to the method previously described.

Bacteriological examination. Both aerobic and anaerobic cultures were made of the solutions prior to their coagulation by Dr. J. Bronfenbrenner, and we desire to express our appreciation for his assistance. No bacterial growth was obtained in either instance after forty-eight hours.

EXPERIMENT 1. Methyl glyoxal and leucocytes. About 25 cc. of washed leucocytes were suspended in 100 cc. Henderson phosphate solution with 3 grams of calcium phosphate and about 1 gram methyl glyoxal, and allowed to stand for eighteen hours at 37° . There was obtained 0.283 gram zinc lactate.

0.0511 gram of the recrystallized anhydrous salt after ignition gave 0.025 gram ZnO = 33.50 per cent ZnO. Calculated = 33.40 per cent.

0.2540 gram zinc lactate in 5 cc. water, total weight 5.3623 grams, gave rotation in a 2 dm. tube of $\alpha = -0.14^{\circ}$ (pure D-light).

EXPERIMENT 2. Methyl glyoxal and kidneys. Both kidneys of a rabbit were minced and added to 100 cc. Henderson phosphate solution containing 3 grams of calcium phosphate, and about 1 gram of methyl glyoxal in 10 cc. water added. There was obtained 0.2440 gram zinc lactate.

0.0595 gram of the anhydrous salt after ignition gave 0.0198 gram ZnO = 33.28 per cent ZnO. Calculated = 33.40 per cent.

0.2440 gram zinc lactate dissolved in 5 cc. water, total weight 5.8350 grams, gave a rotation in a 2 dm. tube of $\alpha = 0.10^{\circ}$ (pure D-light).

A CONTRIBUTION TO A THEORY CONCERNING THE INTERMEDIARY METABOLISM OF CARBO- HYDRATES AND PROTEINS.

THE MUTUAL INTERCONVERSION OF α -AMINO-ACIDS, α -HYDROXY-ACIDS AND α -KETONIC ALDEHYDES.

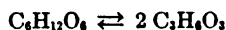
BY H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York.)

(Received for publication, May 2, 1913.)

The object of the following communication is to give a preliminary account of certain new observations that appear to be of significance in connection with the intermediary metabolism of carbohydrates and of proteins.¹

It has long been known that lactic acid occupies an exceptional position in relation to the metabolism of glucose and of amino-acids. Its formation from glucose and glycogen has been accepted for many years and the work of Embden and his collaborators² demonstrating its production during the perfusion of glycogen-rich livers afforded strong support for this view. But the first really convincing evidence of the decomposition of glucose with a formation of lactic acid commensurate with the disappearance of glucose was furnished by the admirable experiments of Levene and Meyer³ on the action of leucocytes upon sugars. The converse change, namely, the conversion of lactic acid into glucose, was proved to occur in the diabetic organism by Mandel and Lusk.⁴ We may therefore represent the change as follows:



It is generally conceded that the change represented above cannot

¹ We shall present the experimental details in a paper to be published immediately.

² Embden and Kraus: *Biochem. Zeitschr.*, xlv, p. 1, 1912.

³ This *Journal*, xi, p. 361, 1912, and later papers.

⁴ *Amer. Journ. of Physiol.*, xvi, p. 129, 1906.

be direct but that intermediate products must take part in the reaction. Embden and his co-workers have considered the possibility of the formation of lactic acid from glyceric aldehyde and from dihydroxyacetone and have been able actually to demonstrate lactic acid production from these substances in the liver. On the other hand we have occupied ourselves with experiments concerning the possibility of lactic acid formation from methyl glyoxal and Levene and Meyer from their work on the action of leucocytes on sugars have been led to the same consideration. The possibility of methyl glyoxal being formed from glucose followed from Pinkus⁵ observation of the formation of methyl glyoxal diphenylhydrazone on treating glucose with caustic soda in the presence of phenylhydrazine. The conditions of this experiment do not of course preclude complicated rearrangements, but recently we have been able to demonstrate the presence of methyl glyoxal in distillates obtained from the simple treatment of glucose with sodium phosphate solution. Moreover, Nef held the view that methyl glyoxal was the precursor of lactic acid in biochemical reactions,⁶ although he apparently abandoned this idea later.⁷

We⁸ have shown that small amounts of tissue extracts, acting under suitable conditions, may transform almost quantitatively many grams of methyl glyoxal into lactic acid in the course of a few minutes, so that no question of possible bacterial action need be considered. We have further shown that blood cells effect the same change, and that the active agent is an enzyme which is active in the intact animal. The enzymes we have named "Glyoxalase."

Since then we have been able to show the formation of abundance of lactic acid on perfusing a surviving liver with blood containing methyl glyoxal. It is a noteworthy fact that in every case we have obtained a mixture of *d* and *l* forms of lactic acid, and, as in our previous paper, we are inclined to believe in the presence of more than one enzyme of the type of glyoxalase.⁹ Later in

⁵ *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 31, 1898.

⁶ *Liebig's Annalen*, cccxxv, p. 279, 1904.

⁷ *Ibid.*, ccclvii, p. 305, 1907.

⁸ This *Journal*, xiv, p. 155, 1913.

⁹ It is noteworthy that Embden, Baldes and Schmitz obtained mixtures of *l* and *d* lactic acid from glyceric aldehyde: *Biochem. Zeitschr.*, xlv, p. 108, 1912.

this paper evidence will be adduced showing that many glyoxals other than methyl glyoxal may be derived from amino-acids so that it is probable that separate catalysts of the type of glyoxalase may be concerned in their metabolism. It should be noted however that in continuation of their previous work Levene and Meyer have succeeded in showing that leucocytes and kidney tissue may convert methyl glyoxal into lactic acid containing an excess of the dextro component, *i.e.*, the lactic acid commonly encountered in animal tissues.¹⁰

Finally we have been able to show that methyl glyoxal and also *l* lactic acid when given to the diabetic animal yield glucose, thus furnishing another link in the chain of reversible reactions. Whether methyl glyoxal is converted into glyceric aldehyde prior to glucose synthesis is at present undecided but appears probable.

There still remained the question, can lactic acid yield methyl glyoxal? While we can hardly hope at present to detect methyl glyoxal in the animal body we are able as the result of new experiments *in vitro* to show that this reaction is readily brought about. In other words, *the conversion of methyl glyoxal into lactic acid is a reversible reaction.*¹¹ It is likely therefore that the action of glyoxalase is also reversible. We were able to demonstrate the conversion of lactic acid into methyl glyoxal by simple digestion of aqueous lactic acid at 37°C. with nitrophenylhydrazine, a substance which forms an extremely insoluble derivative with methyl glyoxal.¹²

We may therefore picture the relationships between glucose, methyl glyoxal and lactic acid as in the following scheme:



But other substances than lactic acid have close metabolic relationships with glucose. Experiments upon glycosuric animals

¹⁰ This *Journal*, p. 551, 1913. Since the appearance of our first communication on glyoxalase, an undated paper by Neuberg has appeared (*Biochem. Zeitschr.*, xlix, p. 502, 1913) in which the conversion of methyl glyoxal into lactic acid is described. Apparently Neuberg was unaware of our earlier work.

¹¹ Cf. *Proc. Chem. Soc.*, 1913, No. 130.

¹² In addition to methyl glyoxal-dinitrophenylhydrazone, α -nitrophenylhydrazino-propionic acid and the nitrophenylhydrazone of pyruvic acid are formed. Their separation however is easy.

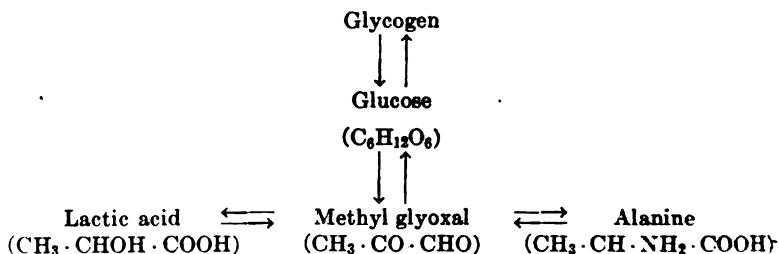
Metabolism of Carbohydrates and Proteins

especially by Lusk and Ringer, and later by one of us¹³ have shown that many amino-acids are apparently readily converted into glucose. In a recent paper it was suggested that methyl glyoxal might be an intermediate product in some cases at least. We have now obtained very definite evidence concerning a possible mode of conversion of an amino-acid such as alanine into sugar.

Every α -amino-acid we have thus far examined¹⁴ has furnished evidence that it may undergo dissociation to a limited extent at low temperatures in feebly acid solution with formation of α -ketonic aldehydes and ammonia.



The demonstration of this reaction was only rendered possible by the use of a substance such as nitrophenylhydrazine which would remove the ketonic aldehyde from the reacting medium as quickly as it was formed. It is an extremely easy matter to prepare methyl glyoxal-dinitrophenylhydrazone from alanine on digesting it at low temperatures with nitrophenylhydrazine, preferably in the presence of a little acetic acid. There is little doubt that the reaction is reversible, so that we are now in a position for the first time to construct an approximate though doubtless incomplete scheme for the interconversion of alanine, lactic acid, methyl glyoxal and glucose by a series of reactions only involving the addition or removal of water or ammonia.



¹³ This *Journal*, xiv, p. 321, 1913; *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

¹⁴ Including glycine, alanine, valine, leucine, aspartic acid and phenyl-alanine. The reaction in the case of glutamic acid is complicated by the fact that, as expected, one of its two carboxyl groups is retained and the product is relatively soluble.

The above scheme is of interest in connection with the mechanisms for the maintenance of approximate neutrality in living cells. It should be noted that alanine may be converted into "neutral" methylglyoxal with liberation of alkaline ammonia, while the methylglyoxal in turn may be converted into acid lactic acid by union with water. Furthermore we have found by experiments *in vitro* that the production of lactic acid from methyl glyoxal is promptly checked unless provision is made for the neutralization of the free acid. In addition we find that the conversion of alanine into methyl glyoxal and ammonia is greatly accelerated by acids. Thus it is clear that we are dealing with an exquisitely delicately adjusted condition of equilibrium provided with automatic checks guarding against undue development of either acid or alkali.

The demonstration of the conversion of amino-acids into α -ketonic aldehydes seems to us to be of importance in many connections. *It appears probable that α -ketonic aldehydes may represent the first step in the metabolism of amino-acids.* This hypothesis furnishes a reasonable explanation of the mechanism of the conversion of α -amino-acids into α -ketonic acids, a reaction so clearly demonstrated to occur in the animal organism by Neubauer,¹⁵ and later by Knoop. It is only necessary to assume the formation of the corresponding α -ketonic aldehyde from the amino-acid and oxidation of the former. Thus we have observed the formation of phenyl glyoxylic acid on perfusing a liver with blood containing phenyl glyoxal.



Furthermore it shows why the corresponding hydroxy-acids are not obligate steps in the catabolism of amino-acids, for it is known, for example, that α -hydroxy-phenylpropionic acid is relatively resistant to change in the body, while phenylalanine and phenylpyruvic acid are readily oxidized.

In addition it indicates a probable mode of synthesis of amino-acids in living cells from nitrogen-free ketonic aldehydes and ammonia.¹⁶ Thus it is known that lactic acid and alanine are inter-

¹⁵ *Deutsch. Arch. f. klin. Med.*, xcv, p. 211, 1909; *Zeitschr. f. physiol. Chem.*, lxvii, p. 489, 1910; lxxi, p. 153, 1911.

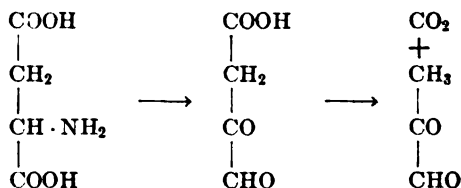
¹⁶ We are at present engaged on investigations of this character.

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convertible in the animal body.¹⁷ The scheme on p. 558 clearly indicates a probable mechanism for the reaction.

The formation of α -ketonic aldehydes from amino-acids has an interesting connection with the synthesis of pyrimidine derivatives in the body, as was pointed out in the case of methyl glyoxal derivable from glucose by Knoop and Windaus.

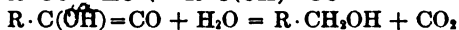
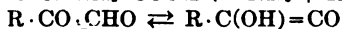
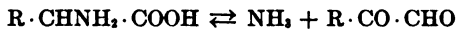
Again, the formation of glucose from various amino-acids in the diabetic organism is rendered much more intelligible. The conversion of alanine into glucose has already been referred to. The case of aspartic acid is particularly instructive. Lusk and Ringer have shown that three out of the four carbon atoms of aspartic acid are convertible into glucose, and this is precisely what would be expected on the basis of β -ketonic aldehyde acid formation, for the substance formed, being a β -ketonic acid, would undoubtedly lose carbon dioxide, yielding methyl glyoxal. We have had no difficulty in actually demonstrating the conversion of aspartic acid into a glyoxal-dinitrophenylhydrazone *in vitro*. Aspartic acid is therefore at once brought into biochemical relation with glucose, lactic acid and alanine.



Finally the relation of α -ketonic aldehydes to alcohol formation from amino-acids and from sugar may be mentioned. Buchner and Meisenheimer's original experiments on the fermentation of methyl glyoxal are unconvincing in the light of present knowledge, since we have shown that much acid may be formed from glyoxals by the action of glyoxalase present in the yeast cells. New experiments are undoubtedly necessary. In the meantime it would seem a more attractive hypothesis to assume that the conversion of amino-acids into alcohols as observed by Felix Ehrlich proceeds

¹⁷ Neuberg and Langstein: *Arch. f. (Anat. u.) Physiol.*, Suppl. Band. 1903. p. 514; Embden and Kraus: *loc. cit.*

by way of the ketonic aldehydes rather than by the hydroxy-acids as has been assumed.



We are at present engaged in testing this hypothesis experimentally.

SUMMARY.

1. It is shown that by a suitable choice of experimental conditions it is possible to convert at low temperatures α -amino- and α -hydroxy-acids into α -ketonic aldehydes. Lactic acid and alanine for example, yield methyl glyoxal.

2. Methyl glyoxal is acted upon by enzymes named "glyoxalases," present in the animal body, with formation of both *d*- and *l*-lactic acid. When given to the glycosuric animal methyl glyoxal and both *d* and *l* lactic acids yield glucose. Methyl glyoxal is believed, therefore, to be an intermediate product in the mutual interconversion of alanine, lactic acid and glucose.

The relationship in the living cell between alanine, lactic acid, methyl glyoxal and glucose is believed to constitute a delicately adjusted equilibrium concerned with the maintenance of approximate neutrality. The view is advanced that α -ketonic aldehydes may represent the first step in the metabolism of amino-acids, and that the formation of these substances furnishes an adequate explanation of the origin of α -ketonic acids from α -amino-acids. Furthermore it affords an explanation of the fact that α -hydroxy-acids are not obligate steps in the catabolism of most α -amino-acids.

The relation of α -ketonic aldehyde formation is discussed in connection with the mechanism of glucose production in the glycosuric animal, with the synthesis of pyrimidine derivatives, and in connection with the mechanism of alcoholic fermentation.

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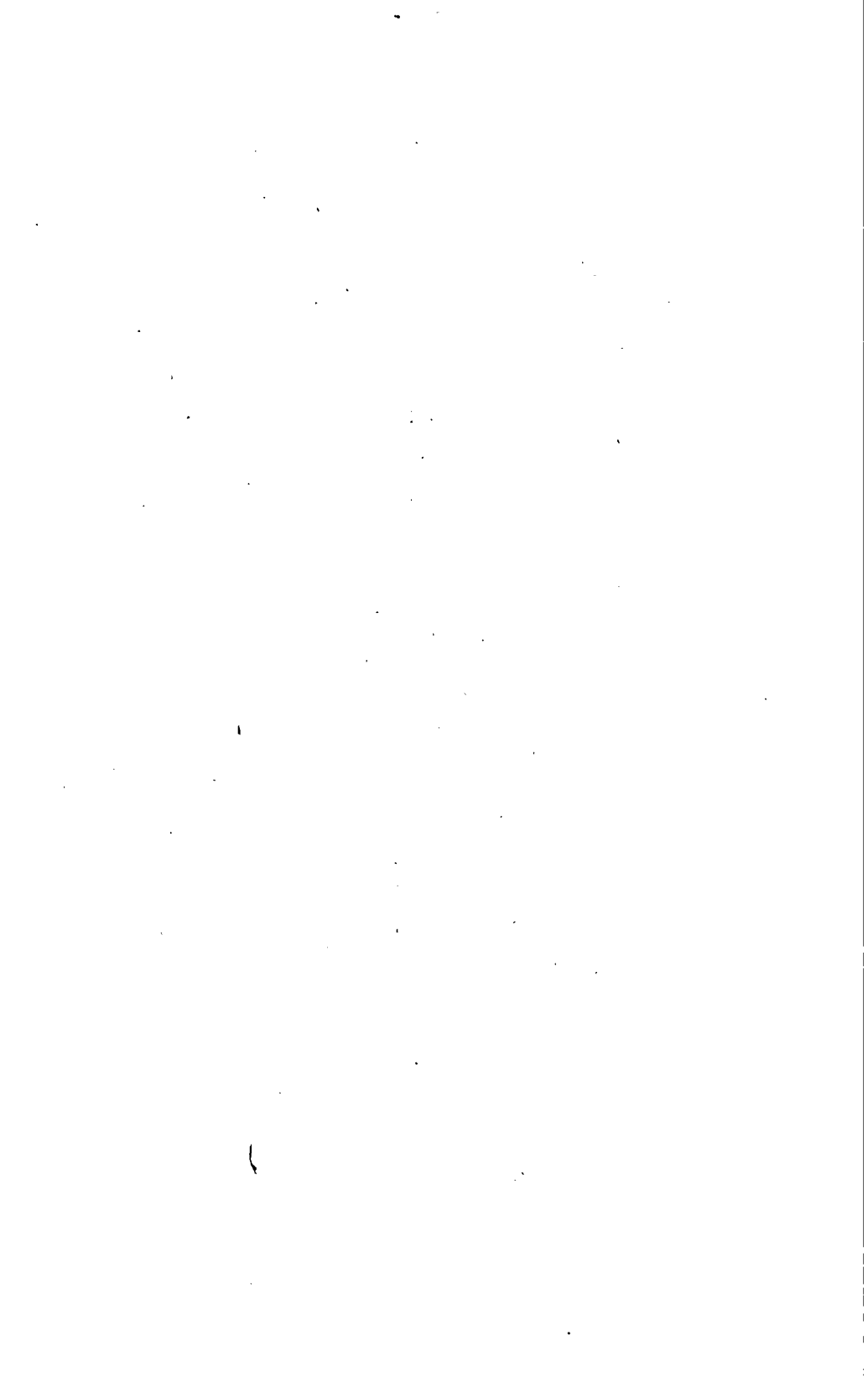
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